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ERRATA

The Journal of Nutrition, vol. 39, no. 2, October, 1949.

HEBER R. BALDWIN AND CLARENCE P. BERG

The influence of optical isomerism and acetylation upon the availability of tryptophan for maintenance in man.

Page 204, line 28, the word "available" should have been "unavailable." Corrected line reads —

vert the acetyl-D-tryptophan into an unavailable product; one

---

BENJAMIN H. ERSHOFF

Protective effects of soybean meal for the immature hyperthyroid rat.

The word "reduction" appearing in line 4, page 279 under Summary should have been "elevation." The corrected line reads —

the attendant elevation in basal metabolic rate.

ASCORBIC ACID METABOLISM OF OLDER ADOLESCENTS [1]

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ONE FIGURE

(Received for publication April 18, 1949)

INTRODUCTION

A review of the literature in the field of ascorbic acid requirements showed few data on adolescent subjects. The recommended allowances for ascorbic acid for adults, children 8 to 12 years of age, pre-school children and infants were derived from experimental data on those age groups (National Research Council, '45). The allowances for children over 12 years of age apparently were not based on experimental studies on this age group but were derived from data obtained from studies on adults and younger children.

So far as the present authors know, the first controlled study on the vitamin C requirements of adolescents was re-

[1] Published as Technical Paper No. 578 with the approval of the Director of the Oregon Agricultural Experiment Station. Contribution of the Nutrition Laboratory of the Home Economics Experiment Station in cooperation with the School of Home Economics, Oregon State College, and the Bureau of Human Nutrition and Home Economics, U. S. Department of Agriculture.

[2] The data in this paper are taken from the thesis presented by Bessie L. Davey to the Graduate School faculty of Oregon State College in partial fulfillment of the requirements for the Ph.D. degree, June, 1949. Further details will be found in the thesis.

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ported from this laboratory (Storvick et al., '47). Eight young adolescents, 4 boys and 4 girls who were 12 through 14 years of age, participated in that study, in which fasting plasma ascorbic acid determinations were made daily during three one-week periods when the ascorbic acid intake was controlled. For these subjects it was found that the daily allowance of ascorbic acid recommended by the National Research Council ('45) resulted in plasma ascorbic acid values lower than those which they attained during the saturation period but well above 0.6 mg%, the quantity considered adequate by Butler ('40).

In order to extend the knowledge of ascorbic acid requirements to the older adolescent age group, studies were conducted in which 16 Oregon State College freshmen, 16 through 19 years of age, participated as subjects.

The various levels of ascorbic acid intake for this study were selected in order to determine: (1) the concentrations of ascorbic acid in the plasma of subjects receiving the National Research Council recommended allowance for ascorbic acid, and (2) whether or not a small decrease in ascorbic acid intake, i.e., 10 mg less than the recommended allowance, had a significant effect on the concentration of ascorbic acid in the plasma.

EXPERIMENTAL

The work reported in this paper was done during the academic years 1946 to 1948. The studies during the first year were divided into 4 periods of 7 days each in order to compare the daily values of plasma ascorbic acid when the subjects received the following: first period, unrestricted diets; second period (or saturation period), a daily supplement of 200 mg of crystalline ascorbic acid [3] in addition to the ascorbic acid in the food; third period, the total daily ascorbic acid intake recommended by the National Research Council; and 4th period, a total intake of 10 mg less than they received during the third period. During the second year the studies were

[3] Acknowledgment is made to Merck and Company Inc., Rahway, New Jersey, for a generous supply of crystalline ascorbic acid.

divided into three periods of 10 days each, allowing more time to adjust to each level of ascorbic acid intake. The daily ascorbic acid intakes for the three periods were the same as those for the second, third and 4th periods, respectively, of the preceding year's studies.

TABLE 1

Description of subjects

YEAR		SUBJECT	AGE	MEAN WEIGHT	HEIGHT
				lb.	in.
1946–47	Girls	MG	18	119	61½
		WH	16	125	67
		BR	19	130	65½
		PS	18	111	59½
	Boys	JC	18	155	71½
		VD	18	156	69½
		JJ	18	157	71
		GS	18	170	71½
1947–48	Girls	NA	18	117	65½
		BD	18	116	65
		MF	18	145	65½
		RR	18	153	64½
	Boys	TC	18	186	73½
		DE	18	182	73½
		WP	18	129	66
		DR	18	151	70½

The ages, weights, and heights of the human subjects are given in table 1. These individuals were fed in a college dormitory. All of the foods which they ate were weighed and recorded. The general plan for controlling the intake of ascorbic acid from food was the same as that described in the previous paper by Storvick et al. ('47). Samples of foods which might be presumed to contain ascorbic acid were analyzed [4] by the method of Loeffler and Ponting ('42). Through-

[4] Details of the experimental methods and procedures are available in Technical Bulletin 12 (1947) published by the Oregon Agricultural Experiment Station and from the thesis submitted to the Graduate Faculty of Oregon State College by Bessie L. Davey ('49).

out the experimental studies blood samples were taken daily before breakfast and the reduced ascorbic acid in plasma was determined by the micro method of Farmer and Abt ('36).

RESULTS

Table 2 presents a summary of the means for plasma ascorbic acid concentrations for each subject. During the 1946–47 studies the plasma values for all of the days in the first, or orientation, period of each study were included in the calculations, since no attempt was made to control the ascorbic acid intake for this period. For the second, third and 4th periods the plasma values were averaged for the last 5 days, thus allowing two days for the subjects to adjust to the new levels of intake. In the 1947–48 studies the mean values were calculated both on the basis of the last 8 days and the last 5 days, allowing two and 5 days, respectively, for adjustment to new levels. By comparison of these two sets of calculations we hoped to ascertain whether the longer experimental periods were really necessary. All of the daily ascorbic acid intake values were averaged in every experimental period.

The significance of the differences between the means was tested statistically. The difference is considered significant if it is twice as large as the standard deviation of the differences.

Girls

In the first, or orientation, period of the girls' study in 1946–47, three of the 4 girls showed near-saturation values according to the results of fasting plasma ascorbic acid determinations (fig. 1A). The mean fasting plasma ascorbic acid values were 1.00 mg% or higher for three of the subjects during this period.

A rise in plasma ascorbic acid values was observed in all cases with the ingestion of saturation levels of ascorbic acid during the second period. Although three subjects appeared to be "saturated," WH may possibly not have been, since on

TABLE 2

Summary of mean plasma ascorbic acid concentrations and deviations from the mean for each subject during experimental periods on different levels of intake

SUBJECT	UNRESTRICTED	"SATURATION"		NRC RECOMMENDED ALLOWANCE		10 MG LESS THAN NRC RECOMMENDED ALLOWANCE	
	All days	Last 8 days	Last 5 days	Last 8 days	Last 5 days	Last 8 days	Last 5 days
	mg %	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*
Girls							
1946–47							
M.G.	1.01 ± 0.17		1.13 ± 0.03		0.99 ± 0.10		0.86 ± 0.07
W.H.	0.61 ± 0.14		1.11 ± 0.11		0.96 ± 0.07		0.80 ± 0.05
B.R.	1.00 ± 0.12		1.15 ± 0.04		0.97 ± 0.03		0.92 ± 0.06
P.S.	1.08 ± 0.10		1.21 ± 0.06		1.07 ± 0.08		1.04 ± 0.10
1947–48							
N.A.		1.03 ± 0.05	1.02 ± 0.07	0.85 ± 0.07	0.83 ± 0.08	0.81 ± 0.05	0.82 ± 0.04
B.D.		1.18 ± 0.07	1.19 ± 0.06	0.88 ± 0.06	0.90 ± 0.05	0.91 ± 0.07	0.95 ± 0.06
M.F.		1.25 ± 0.12	1.26 ± 0.10	0.97 ± 0.08	0.98 ± 0.04	0.96 ± 0.02	0.96 ± 0.02
R.R.		1.22 ± 0.12	1.27 ± 0.06	1.03 ± 0.04	1.02 ± 0.04	0.98 ± 0.04	0.99 ± 0.04
Boys							
1946–47							
J.C.	0.78 ± 0.07		1.07 ± 0.10		0.91 ± 0.06		0.79 ± 0.07
V.D.	0.39 ± 0.11		1.01 ± 0.09		0.82 ± 0.04		0.75 ± 0.07
J.J.	0.39 ± 0.04		0.90 ± 0.11		0.90 ± 0.05		0.80 ± 0.07
G.S.	0.61 ± 0.13		1.07 ± 0.03		0.86 ± 0.04		0.84 ± 0.04
1947–48							
T.C.		0.80 ± 0.44	0.99 ± 0.20	0.90 ± 0.06	0.90 ± 0.02	0.82 ± 0.09	0.84 ± 0.05
D.E.		0.71 ± 0.32	0.88 ± 0.10	0.69 ± 0.10	0.67 ± 0.04	0.67 ± 0.03	0.68 ± 0.02
W.P.		1.11 ± 0.11	1.12 ± 0.09	0.79 ± 0.09	0.81 ± 0.04	0.72 ± 0.07	0.74 ± 0.06
D.R.		1.04 ± 0.17	1.09 ± 0.06	0.73 ± 0.09	0.69 ± 0.02	0.73 ± 0.09	0.74 ± 0.08

the last day of the saturation period her plasma value was the highest noted. There is a question, therefore, as to whether a further increase would have resulted if the period had been extended.

There was a statistically significant decrease in the plasma ascorbic acid concentration in all subjects when the mean level of ascorbic acid intake was decreased for the third period to 82 mg, which is slightly higher than the 80 mg

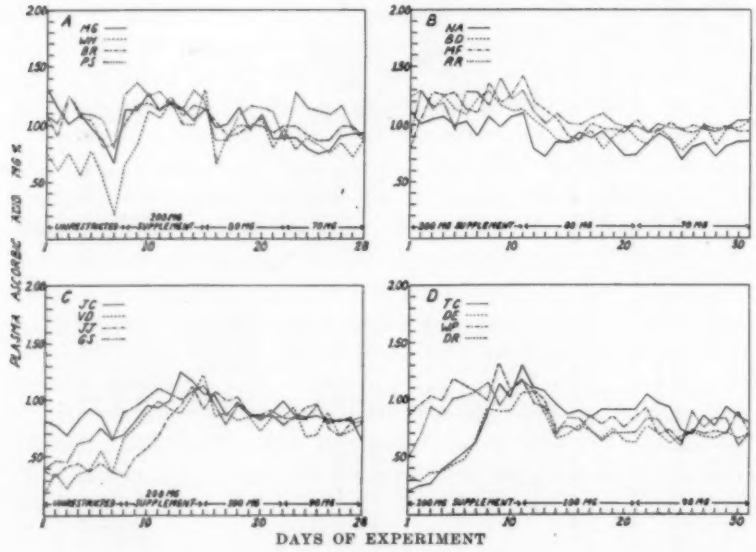

Fig. 1. Daily plasma ascorbic acid values for all subjects.

allowance recommended by the National Research Council. The mean plasma values on this level of intake were very similar to those for each subject during the first or unrestricted period, with the exception of WH, whose value was 0.35 mg% higher than her mean value for the first period. WH's mean plasma value was, however, very similar to those of the other subjects during the third period.

A further statistically significant decrease in plasma ascorbic acid values was shown by two subjects (MG and WH)

during the 4th period when the mean ascorbic acid intake was 71 mg, or 11 mg less than during the third period.

The 10-day saturation period during the 1947–48 study seems to have been sufficiently long for the plasma to become saturated in all 4 girls, as is indicated by a "leveling off" at plasma values above 1.00 mg% (fig. 1B).

A statistically significant decrease in plasma ascorbic acid between the saturation period and the second period, when the mean ascorbic acid intake was 80 mg per day, was shown by all subjects, regardless of whether the plasma values of the last 8 days, or only the last 5 days, of the period were considered.

With these 4 subjects a 10 mg decrease in the level of ascorbic acid intake (to a mean of 70 mg per day) resulted in no statistically significant decrease in the plasma ascorbic acid values when calculated on the basis of the last 5 days, and in only one case (RR) was there a significant decrease when they were calculated on the last 8 days of the period.

Boys

The fairly low mean plasma ascorbic acid values of the boys in the first period of the 1946–47 study, when the ascorbic acid intake was not restricted, were a general indication of previously low intakes (fig. 1C). A marked rise in plasma ascorbic acid was observed in three of the subjects, JC, VD and GS, when 200 mg doses of ascorbic acid were given as supplements during the second or saturation week. Although the mean ascorbic acid intake during this saturation period was very high, 319 to 357 mg per day, it was questionable whether the subjects were "saturated" at the end of the week, particularly VD and GS, whose values were increasing day by day. A steady but more gradual rise was shown by JJ during this period and his mean value for the period was considerably lower than those of the other subjects. On the 4th day of the saturation period JJ reported to the Student Health Service at the College, where he was given a series of injections for poison oak. JJ remained on

the experiment as a matter of interest although his data cannot be considered those of a normal subject and were not included in the statistical analyses.

During the third week 100 mg of ascorbic acid, the recommended allowance of the National Research Council for this particular age, were given to the subjects. A marked downward trend was noticed in the plasma values at this time. The decrease in mean plasma values from the saturation period to the period of the 100 mg mean intake was statistically significant for JC, VD and GS.

In the 4th period, when the ascorbic acid intake was decreased 10 mg below the level of intake during the third period, a further significant decrease in plasma ascorbic acid was shown only by JC. The plasma concentration of GS appeared to have reached a plateau by the end of the period at this level of intake, but whether the plasma values of the other subjects had reached plateaus was not so clearly evidenced.

In the 1947–48 study the concentrations of ascorbic acid in the plasma samples of WP and DR were indicative of saturation by the end of the 10-day saturation period (fig. 1D). At the beginning of the study TC and DE had very low fasting plasma ascorbic acid concentrations, 0.21 and 0.34 mg%, but throughout the saturation period their plasma values continued to rise. The mean value for TC calculated on the data for 8 days was 0.80 mg%, but his mean value was 0.99 mg% when only the last 5 days were considered. For DE a similar difference in the means was observed, i.e., 0.71 mg% on the data for 8 days compared to 0.88 mg% on a basis of 5 days. From the plasma values of the last few days of this period it would appear that these subjects were approaching saturation, but we cannot say that they were definitely saturated.

When the intake of ascorbic acid was decreased to 100 mg in the second period, a statistically significant lowering of the mean fasting plasma ascorbic acid values occurred in all cases when calculated on the basis of the last 5 days of the

experimental period. Calculated on the basis of data from the last 8 days of the period, only two of the subjects (WP and DR) showed a statistically significant decrease in the mean fasting plasma ascorbic acid content. The differences between the means of plasma ascorbic acid values were not statistically significant for TC and DE, the two subjects who started the study with very low values.

A further decrease in the ascorbic acid intake to 90 mg made no statistically significant differences in the mean fasting plasma ascorbic acid concentrations of the 4 subjects when calculated on the basis of the last 5 days of the experimental period. The calculations based on the last 8 days revealed inconsistent results, TC and WP showing a significant decrease between the means whereas DE and DR did not.

In addition to the above-mentioned analysis of the results, the data of both years were also analyzed statistically for variance (Snedecor, '46).[5] A statistically significant difference was found between the means of the saturation period and the period on the recommended allowance of the National Research Council, while the difference in the means between the period on the recommended allowance and the period of 10 mg less than this amount was not statistically significant for the boys but was significant for the girls. It was interesting to note that although the girls were ingesting 20 mg of ascorbic acid less than the boys during comparable periods, they had significantly higher mean plasma ascorbic acid values.

The results of this study were similar to those obtained with the younger adolescent group; that is, the daily allowance of ascorbic acid recommended by the National Research Council did not maintain the plasma values at as high levels as were reached during the saturation period. In 22 of the 24 subjects studied in this laboratory the National Research Council recommended allowance maintained mean plasma

[5] The authors are indebted to Dr. J. C. R. Li, Assistant Professor of Mathematics, for assistance in the analysis of variance.

values of above 0.80 mg% under the conditions of our studies. The two exceptions were older adolescent boys.

SUMMARY

1. The recommended allowance of the National Research Council, 100 mg for the 18-year-old boys and 80 mg for the 16- to 19-year-old girls, did not maintain mean plasma values at levels as high as their respective saturation means. For the girls all the mean values were above 0.80 mg%, ranging from 0.83 to 1.07. The boys' values ranged from 0.67 to 0.91 mg%; two out of the 7 values were below 0.80 mg%.

2. When the ascorbic acid intake was decreased to 10 mg less than the recommended allowance of the National Research Council, it was found that for 6 of the 8 girls the 70 mg intake of ascorbic acid was as effective as the 80 mg intake in maintaining the ascorbic acid concentration of the plasma, and that for 6 of the 7 boys an intake of 90 mg of ascorbic acid was as effective as 100 mg in maintaining the plasma ascorbic acid concentration (JJ's values were excluded).

3. The 10-day experimental periods were more desirable than the periods of one week. This was particularly true for the saturation period when some of the subjects had been on diets low in ascorbic acid prior to the study.

4. The data in this study were analyzed statistically by testing the significance of differences betweens means and by analysis of variance.

LITERATURE CITED

BUTLER, A. M. 1940 Report of the Committee on Vitamins of the American Academy of Pediatrics, p. 14.

DAVEY, BESSIE L. 1949 The Ascorbic Acid Requirements of Older Adolescents. Thesis, Oregon State College.

FARMER, C. J., AND A. F. ABT 1936 Determination of reduced ascorbic acid in small amounts of blood. Proc. Soc. Exp. Biol. Med., *34:* 146–150.

LOEFFLER, A. J., AND J. D. PONTING 1942 Ascorbic acid. Rapid determination in fresh, frozen or dehydrated fruits and vegetables. Ind. Eng. Chem., Anal. Ed., *14:* 846–849.

NATIONAL RESEARCH COUNCIL 1945 Committee on Food and Nutrition. Recommended Dietary Allowances. Revised. Reprint and Circular Series, No. 122, p. 12.

SNEDECOR, GEORGE W. 1946 Statistical Methods. Iowa State College Press, Ames, Iowa, 4th ed.

STORVICK, C. A., M. L. FINCKE, J. P. QUINN AND B. L. DAVEY 1947 A study of ascorbic acid metabolism of adolescent children. J. Nutrition, *33:* 529–539.

MAGNESIUM DEFICIENCY IN THE CHICK

I. CLINICAL AND NEUROPATHOLOGIC FINDINGS [1]

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EIGHT FIGURES

(Received for publication March 30, 1949)

Magnesium has been shown to be an essential constituent of the diet for the mouse (Leroy, '26), the rat (Kruse et al., '32) and the dog (Orent et al., '32). Almquist ('42) reported that magnesium deficient chicks frequently passed into a convulsive state which sometimes terminated fatally. Duncan et al. ('35) have demonstrated that calves may succumb to magnesium tetany at almost any time after 30 days of age.

When an attempt was made to rear baby chicks on highly purified diets (Bird, '45), a peculiar disorder appeared which indicated that at least one essential nutrient was not adequately supplied by the ration. A subsequent report (Bird, '46) indicated that the symptoms of incoordination and convulsions which were found associated with a cerebellar lesion in chicks resulted from a suboptimum level of dietary magnesium. The present report describes both the syndrome which appeared in the chick and the neuropathology resulting from a deprivation of dietary magnesium.

[1] From a thesis submitted to the Graduate Division of the University of California in partial fulfillment of the requirements for the degree of Doctor of Philosophy granted in June, 1948.

[2] Present address: Eastern States Farmers' Exchange, Inc., Westbrook Laboratory, R.F.D. 3, Rockville, Connecticut.

EXPERIMENTAL METHODS AND RESULTS

Single-comb white Leghorn chicks were used in these studies. They were removed from the incubators when one day old and placed in electrically heated metal battery brooders equipped with raised wire-screen floors. Either immediately upon hatching or after they were fed a commercial-type chick starter ration for 7 days, the chicks were wing-banded and segregated into experimental groups of equal weight and vigor. The diets and water were fed ad libitum. The composition of the diets and the histological procedures employed are given in the appendix to this paper.

Clinical symptoms

Chicks receiving the purified-type of diet, 3A, exhibited symptoms indicative of functional disturbance; i.e., poor growth and feathering, decreased muscle tone, squatting, ataxia, fine palpable tremors, forced movements, progressive incoordination of movements, convulsions, and death.

Incidence. Signs indicative of the above condition were not observed in all of the chicks fed diet 3A. The incidence, based upon the number of chicks surviving the experimental period, varied from 63 to 100% (table 1). In the 25 experimental groups, 154 chicks or 93% of the 166 chicks which survived the experimental period exhibited signs of functional disturbances. It can be observed in table 1 that the incidence was not influenced by season. This table also shows that the addition of 0.3% of magnesium sulphate to diet 3A gave complete protection against the appearance of deficiency symptoms.

Growth. The data in table 1 indicate that diet 3A had an adverse effect on growth. However, the addition of 0.3% of magnesium sulfate (group 160) produced a growth response, even though it was subnormal.

Functional disturbances. Symptoms of functional disturbances first appeared after the chicks had been fed diet 3A for 7 days. It was first noted that these chicks squatted more

TABLE 1

The incidence of symptoms, the number of survivors, and the growth of groups of chicks fed the magnesium deficient diet and the commercial-type control ration

GROUP NO.	DIET [1] NO.	CHICKS STARTED			CHICKS SURVIVING THE EXPERIMENTAL PERIOD					
		Date	Age	no.	Total no.	With symptoms		Weight gain /bird		
						no.	%	7 days	14 days	21 days
			days					*gm*	*gm*	*gm*
10	3A	Feb.	7	8	6	6	100	15	18	..
12	3A		7	7	6	5	83	13	17	..
23	CR	1944	7	7	7	0	0	39	85	..
24	3A	Mar.	7	8	6	5	83	11	..	..
27	3A		7	8	8	6	75	12	..	..
40	CR	1944	7	7	7	0	0	33	..	..
45	3A		7	9	8	8	100	33	66	..
47	3A	April	7	10	10	10	100	23	50	..
50	3A	1944	7	10	9	9	100	24	47	..
62	CR		7	10	10	0	0	40	93	..
68	3A	July	7	10	9	9	100	21	41	63
73	CR	1944	7	10	10	0	0	30	80	139
76	3A	Dec.	7	9	8	5	63	16	37	60
81	CR	1944	7	9	9	0	0	31	87	140
84	3A	Feb.	7	10	10	10	100	16	37	..
88	CR	1945	7	9	9	0	0	17	56	..
93	3A	Aug.	1	10	6	6	100	11	17	..
96	3A		1	10	9	9	100	13	21	..
101	CR	1945	1	10	10	0	0	10	41	..
106	3A	Aug.	7	5	5	4	80	23	43	..
111	CR	1945	7	5	5	0	0	22	55	..
112	3A	Sept.	7	13	13	13	100	23	50	76
114	3A		7	13	12	12	100	30	59	83
115	CR	1945	7	13	13	0	0	35	75	136
120	3A	Oct.	7	7	7	7	100	25	44	..
129	CR	1945	7	7	7	0	0	38	98	..
133	3A	Dec.	7	9	7	7	100	22	45	74
135	3A		7	9	8	7	85	29	45	68
140	CR	1945	7	9	8	0	0	29	80	149
141	3A	Feb.	1	16	12	10	83	15	24	28
153	CR	1946	1	16	16	0	0	15	43	95
154	3A	Mar.	1	10	7	6	85	13	12	14
160	3J		1	10	9	0	0	18	38	65
165	CR	1946	1	10	10	0	0	12	39	87
Total	3A			191	166	154	93			
	CR			122	121	0	0			

[1] 3A Purified-type of diet deficient in Mg, composition given in appendix.
3J Diet 3A plus 0.3% magnesium sulphate.
CR Commercial-type control ration.

than did those birds fed the commercial-type ration. Shortly thereafter a peculiar gait was observed, which could be characterized by the term "goose-step." This became progressively worse until severely affected chicks could take only a few staggering steps. In handling these chicks, a loss in muscle tonus and the presence of fine, palpable, tonoclonic spasms of the body muscles were noted. Complete paralysis of the leg muscles did not occur, since a strong stepping reflex could be elicited even though a standing position was untenable.

Convulsions were observed in many of the affected birds. These attacks appeared suddenly and unpredictably with no correlation between the severity of the clinical signs and the appearance of convulsions. A bird in a convulsive attack would exhibit propulsive or retropulsive movemnts, and would fall on its side with its legs fully extended and with its head retracted with or without twisting. If the chick did not succumb during the attack, it relaxed within 10 to 15 seconds and remained in a comatose state for several minutes, after which it recovered. Two or three separate convulsions have been observed in the same bird on successive days.

Neuropathology of magnesium deficiency

Gross tissue changes. The chick brains when removed from the cranial fossa showed no evidence of gross pathologic changes.

Microscopic tissue changes. Microscopic tissue changes found in the cerebellums of magnesium deficient chicks consisted of degenerative alterations in the Purkinje cells which comprised swelling, nuclear changes, tigrolysis, and alterations in the staining characteristics of the dendrites. No microscopic changes were noted either in the medullary substance or in the cells of the granular and molecular layers. Those portions of the deep cerebellar nuclei visible in these sections were normal.

An increase in the size of the Purkinje cells was one of the striking consequences of magnesium deficiency. This may be noted in plate 1. The diameters of the Purkinje cells were measured in both the normal and deficient chicks (see appendix). The values obtained from these measurements are tabulated in table 2, together with an analysis of variance to demonstrate the effect exerted on them by diet, age, and

TABLE 2

The mean size and the analysis of variance of Purkinje cells from magnesium deficient and control chicks which have been fed the experimental diets for two- and 4-week periods

MEAN SIZE

Chick age	Average diameter of Purkinje cells [1]	
	Diet 3A	Commercial ration
	μ	μ
2 weeks	17.1 ± 1.4	15.2 ± 1.2
4 weeks	17.8 ± 2.0	15.1 ± 1.4

ANALYSIS OF VARIANCE

Source of variance	sq.	df	m.s.	F.
Total	1,113.22	299	3.72	
Between diets	403.07	1	403.07	169.35 [2]
Within diets	710.15	298	2.38	
Between ages on same diet	16.14	2	8.07	3.45
Within ages on same diet	694.01	296	2.34	
Between chicks of same age on same diet	16.13	8	2.01	1.17
Within chicks of same age on same diet	677.88	288	2.35	

[1] Seventy-five cells were measured in each group.
[2] Highly siginificant at 1% level.

error. As can be seen, there is a measurable increase in the Purkinje cell size in the magnesium deficient animals. The average diameter of these cells was 2.4 μ larger than the average diameter of the normal cells. The analysis of variance clearly indicates that the swelling of the Purkinje cells observed in magnesium deficiency was a true increase, and that this increase was due to the diet fed, since the effects of age and of error were negligible.

Under the low power magnification of the microscope (120 ×) the Nissl substance in normal Purkinje cells appeared to fill the cell body and to project as a point into the cell dendrites (fig. 1, plate 1). Under higher magnification (528 ×), the Nissl substance was observed to be composed of discrete bodies (fig. 3, plate 2). These bodies appeared as rounded or elongated or ellipsoid masses which overlapped one another. They were more numerous around the nucleus and in the region adjacent to the cell membrane. A narrow zone midway between the nucleus and the cell membrane was devoid of Nissl substance. The nucleus was capped by Nissl substance, which projected into the dendrites.

Apparent changes in the Nissl substance due to magnesium deficiency could be detected under low power magnification (fig. 2, plate 1). The Nissl substance did not appear as intensely stained as it did in the normal Purkinje cells. This might have been due in part to the swelling, which pushed the Nissl concretions away from one another and brought more cytoplasm into view. That portion of the Nissl substance which normally projected into the dendrites was not visible.

Under higher magnification it was observed that the Nissl substance itself was altered by a magnesium deficient diet (fig. 4, plate 2). Within any one microscopic field, Purkinje cells were observed with the Nissl substance in various stages of disintegration—ranging from a slightly greater than normal dispersion of the tigroid bodies to their complete disappearance. Ghost-like cells were visible which showed only the outlines of the cell and of its nucleus.

A change in the staining characteristics of the Purkinje cell dendrites was noted in the magnesium deficient chick. This change was brought out in sections stained by the Bodian protargol impregnation technique. A photomicrograph of a normal cerebellar section stained by this procedure is shown in figure 7, plate 3, where one may observe the ramification of the Purkinje cell dendrites out to the very edge of the molecular layer. In the cerebellums from the magnesium de-

ficient chicks the Purkinje cell dendrites and their associated climbing fibers were not stained by the protargol impregnation (fig. 8, plate 3), whereas the basket cells and their axons were stained.

Nuclear changes were observed in the Purkinje cells in magnesium deficiency. The Bodian protargol impregnated sections best demonstrated these changes, although they were visible in sections stained with hematoxylin and eosin (figs. 5 and 6, plate 2). In the protargol preparations the normal Purkinje cell nucleus appeared as a slightly ovoid mass of finely granular material which contained one or two nucleoli (fig. 7, plate 3). In contrast, the nuclear material in the magnesium deficient animals contained lighter stained areas, was composed of larger granules and appeared to be pulled away from the nuclear membrane (fig. 8, plate 3).

DISCUSSION

The form and character of the Nissl substance have been used in the diagnosis of neuropathologic processes ever since Nissl ('03) published his classification of nerve cells based upon Nissl substance morphology. Even though formed Nissl granules have not been satisfactorily demonstrated in living cells (Penfield, '32), and even though the validity of diagnoses based upon changes in Nissl substance morphology has, at times, been seriously questioned, it is generally accepted that changes in the health or vigor of the nerve cells are reflected by changes in the appearance of the Nissl granules. Both Einarson ('35) and Kappers et al. ('36) believe that these granules and their reaction to toxins and fatigue establish the existence of a substance within the living cell, vital to its effective functional activity, which is indicated—although not directly represented—by the stainable Nissl substance of fixed material.

Cowdry (see Kappers et al., '36) has indicated that differences in the size and in the appearance of the Nissl substance is to be expected in similarly fixed material and even in cells side by side in the same material. Differences in the

size of nerve cells of the same type have also been demonstrated. It cannot be said that abnormal Purkinje cells were never seen in the normal cerebellums under discussion, or that Purkinje cells approaching normal limits in size and staining characteristics were never seen in the magnesium deficient chicks. That this is not a serious objection is well exemplified by the measurements of cell size given in table 2. Here it is shown that there was some overlapping of the upper distribution of cell measurements for the normal cells and the lower distribution for the abnormal cells. Their overlapping was of little practical significance, however, since the results obtained by measuring a relatively few numbers of Purkinje cells were highly significant. Differences in Nissl staining were also observed within any one section, supporting Cowdry's observation. In normal cerebellar sections some Purkinje cells were observed in which the Nissl substance was apparently undergoing changes similar to those seen in the magnesium deficient cerebellums. However, these abnormal-type cells seen in normal brains were few in number and scattered. On the other hand, only a few normal-type Purkinje cells were seen in the magnesium deficient animals.

The clinical symptoms observed in the magnesium deficient chicks can be correlated very well with the microscopic pathology. It is well known that cerebellar disease may be manifested by such symptoms as marked disturbance of equilibrium with ataxia, falling and staggering gait, incoordination of movements, tremor and hypotonia (Kappers et al., '36; Tilney and Riley, '38; Larsell, '39). These symptoms of cerebellar disease were observed in the magnesium deficient chicks. The mossy and climbing nerve fibers in the cerebellum, though having a different distribution within this organ, conduct impulses from other parts of the central nervous system to the cerebellar cortex and activate the Purkinje cells. The activated Purkinje cells, in turn, conduct the nervous impulse out of the cerebellar cortex and exert effects on other regions of the central nervous system. Thus, disturbance in Purkinje cell function will affect those regions

of the central nervous system under cerebellar control and will be manifested clinically as cerebellar disease. The alterations in the Purkinje cell structure observed in magnesium deficiency indicated functional impairment of these cells. This functional impairment had necessarily become apparent in the clinical symptoms of magnesium deficiency, signs indicative of cerebellar disease.

SUMMARY

The results of clinical and neuropathologic studies of magnesium deficiency in the growing chick have been presented.

Clinically, magnesium deficiency in the chick is characterized by poor growth and feathering, decreased muscle tone, squatting, ataxia, fine palpable tremors, progressive incoordination of movements, convulsions, and death.

A description is presented of the neuropathologic alterations which were found in the cerebellar Purkinje cells of magnesium deficient chicks. These changes were characterized by swelling, by tigrolysis, by nuclear alterations, and by failure of the Purkinje cell dendrites to stain. There was no evidence of gross pathologic changes.

The neuropathology is correlated with the clinical symptoms.

ADDENDUM

Since the publication of this complete study in the thesis submitted to the Graduate Division of the University of California, in partial fulfillment of the requirements for the degree of Doctor of Philosophy granted in June, 1948, a report has been published indicating that similar pathologic changes were observed in the cerebellums of magnesium-deficient rats (Barron et al., '49).

APPENDIX

Composition of the diets. Diet 3A, which supplied only about one-half of the chick's magnesium requirement, was utilized in most of this study. It had the following composition in grams per 100 gm: water-washed casein 22.2, L-arginine monohydrochloride 0.3, glycine 0.9, L-cystine 0.4, calcium gluconate 5.0, cellulose [3] 5.0, soybean oil

[3] Cellu flour purchased from the Chicago Dietetic Supply Company.

3.0, fish oil [4] 0.25, sodium chloride salt mixture [5] 1.0, $Ca_3(PO_4)_2$ 3.5, K_2HPO_4 1.3, KCl 0.3, $MgSO_4 \cdot H_2O$ 0.1, $Na_2SiO_3 \cdot 9H_2O$ 0.25, cholic acid 0.1, choline chloride 0.2 and glucose [6] 56.2. To every 100 gm of the above mixture were added: solubilized liver eluate equivalent to 4 gm of solubilized liver, thiamine hydrochloride 0.5 mg, pyridoxine hydrochloride 0.4 mg, riboflavin 0.5 mg, calcium pantothenate (dextrorotatory) 1.5 mg, nicotinic acid 1.0 mg, 2-methyl-1, 4-naphthohydroquinone diacetate 1.0 mg, alpha-tocopherol 1.0 mg, and biotin [7] 0.01 mg.

The solubilized liver eluate was prepared by extracting 1 kg of solubilized liver [8] 4 times with two-gallon portions of water brought to pH 4 with sulfuric acid. The solids were allowed to separate by gravity and the supernatant fluid was removed by means of a siphon. The clear extract was then treated twice with 250 gm portions of charcoal [9] at pH 4. The charcoal was removed by filtration and eluted with a solution of 60% acetone and 40% water containing 2% concentrated NH_4OH. The removal of the acetone and the concentration of the eluate were effected by distillation *in vacuo*.

Histological methods. Individual chick brains were studied histologically. The chicks were killed by decapitation and their brains removed immediately. The brains were exposed by cutting through the external sagittal groove of the skull and chipping away the bone covering the dorsal and lateral surfaces. After the brains were freed from the ventral surfaces of the cranial fossae by sectioning the cranial nerves, they were immediately placed in the fixing solution. During the process of their removal the brains were subjected to the least possible amount of manipulation and were bathed with an 0.85% solution of sodium chloride to minimize dehydration effects.

In the early phases of this study, Bouin's fluid was employed routinely as a fixing agent. Later, when sections were to be impregnated with silver, the brains were fixed in the following solution:

[4] Fortified sardine oil containing 3,000 I.U. of vitamin A and 400 A.O.A.C. units of vitamin D per gram.

[5] The sodium chloride salt mixture contained 96.471% sodium chloride, 0.49% manganese, 0.1% iron, 0.5% copper, 0.05% zinc, 0.05% aluminum, 0.002% cobalt, and 0.04% iodine.

[6] Cerelose.

[7] The crystalline biotin was donated by Merck and Company, Inc.

[8] Liver fraction "L" donated by Wilson Laboratories, Chicago, Illinois.

[9] Nuchar "C-45" obtained from the Industrial Chemical Sales Co., Covington, Virginia.

37–40% formaldehyde (U.S.P.) 100 ml, distilled water 900 ml, sodium chloride 8.5 gm, and magnesium carbonate 1.0 gm.[10]

The fixed material was embedded in paraffin and parasagittal sections of the brain were cut at 8 μ to include regions of the cerebral cortex, cerebellum, pons, and medulla oblongata.

These sections were stained with hematoxylin and eosin and with toluidin blue by the procedure recommended by McClung ('29). In later studies, sections were also stained by a modified[11] Bodian protargol impregnation technique (Bodian, '36, '37).[12]

The measurements of the Purkinje cells were made on toluidin blue stained sections with a calibrated Spencer ocular drum micrometer. Twelve slides were examined. These included three slides selected at random from each of the following 4 dietary groups: (a) chicks fed diet 3A for two weeks; (b) chicks fed the commercial-type ration for two weeks; (c) chicks fed diet 3A for 4 weeks; and (d) chicks fed the commercial-type ration for 4 weeks. The diameters of 25 consecutive Purkinje cells were measured. The cell diameter parallel to the boundary between the molecular and granular layers was chosen to represent the diameter of the cell because the point of attachment of the dendrite with its cell body could not always be seen, and thus it was not possible to fix accurately the true cell orientation. It was recognized that these measurements did not necessarily represent the true size of the cell. However, since all of the measurements were consistently made in the same way, their use for comparative purposes was considered to be valid.

REFERENCES

ALMQUIST, H. J. 1942 Magnesium requirement of the chick. Proc. Soc. Exp. Biol. Med., *49:* 544.

BARRON, GEORGE P., SIDNEY O. BROWN AND PAUL B. PEARSON 1949 Histological manifestations of a magnesium deficiency in the rat and rabbit. Ibid., *70:* 220.

BIRD, F. H. 1945 A nutritionally produced cerebellar disorder in chicks, not associated with E avitaminosis. J. Biol. Chem., *161:* 747.

———— 1946 Magnesium supplementation in relation to a previously reported cerebellar disorder in chicks. Poultry Sci., *25:* 396.

BODIAN, DAVID 1936 A new method for staining nerve fibers and nerve endings in mounted paraffin sections. Anat. Rec., *65:* 89.

———— 1937 The staining of paraffin sections of nervous tissues with activated protargol. The role of fixatives. Ibid., *69:* 153.

[10] Lyons, W. R. Personal communication.

[11] See footnote 10.

[12] The author wishes to express his appreciation to Mr. Cecil A. Gunns of the Division of Poultry Husbandry for sectioning and preparation of the slides from the fixed material.

DUNCAN, C. W., C. F. HUFFMAN AND C. S. ROBINSON 1935 Magnesium studies in calves. I. Tetany produced by a ration of milk or milk with various supplements. J. Biol. Chem., *108:* 35.

EINARSON, LARUS 1935 Histological analysis of the Nissl-pattern and -substance of nerve cells. J. Comp. Neur., *61:* 101.

KAPPERS, C. U. ARIËNS, G. CARL HUBER AND ELIZABETH C. CROSBY 1936 The Comparative Anatomy of the Nervous System of Vertebrates including Man. The Macmillan Company, New Yory, N. Y., Vol. I.

KRUSE, H. D., E. R. ORENT AND E. V. MCCOLLUM 1932 Studies on magnesium deficiency in animals. I. Symptomatology resulting from magnesium deprivation. J. Biol. Chem., *96:* 519.

LARSELL, O. 1939 Textbook of Neuro-anatomy and the Sense Organs. D. Appleton-Century Co., Inc., New York, N. Y.

LEROY, J. 1926 Nécesseté du magnésium pour la croissance de la souris. Compt. rend. Soc. Biol., *94:* 431.

MCCLUNG, C. E. 1929 Handbook of Microscopical Technique. Paul B. Hoeber, Inc., New York, N. Y.

NISSL, F. 1903 Die Neuronenlehre und ihre Anhänger. G. Fischer, Jena.

ORENT, E. R., H. D. KRUSE AND E. V. MCCOLLUM 1932 Studies on magnesium deficiency in animals. II. Species variation in symptomatology of magnesium deprivation. Am. J. Physiol., *101:* 454.

PENFIELD, WILDER (editor) 1932 Cytology and Cellular Pathology of the Nervous System. Paul B. Hoeber, Inc., New York, N. Y.

TILNEY, FREDERICK, AND HENRY A. RILEY 1938 The Form and Functions of the Central Nervous System. Ibid.

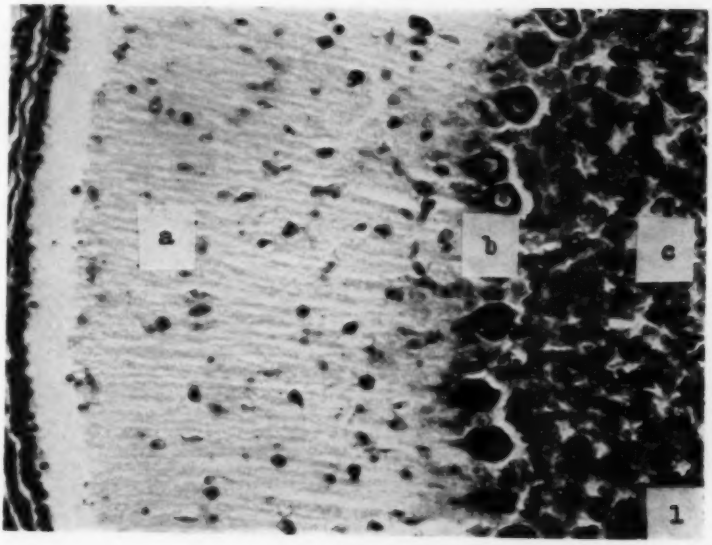
PLATE 1

EXPLANATION OF FIGURES

Photomicrographs of parasagittal sections cut from paraffin embedded chick cerebellums. (a) Molecular layer, (b) line of Purkinje cells, (c) granular layer. Stain, toluidin blue, × 315.

1 From chick no. 2757 fed the commercial-type control ration. The Purkinje cells are of normal size and their Nissl substance has a normal distribution within the cells.

2 From chick no. 2676 fed the magnesium deficient diet 3A. The Purkinje cells are swollen and their Nissl substance shows chromatolysis.


a
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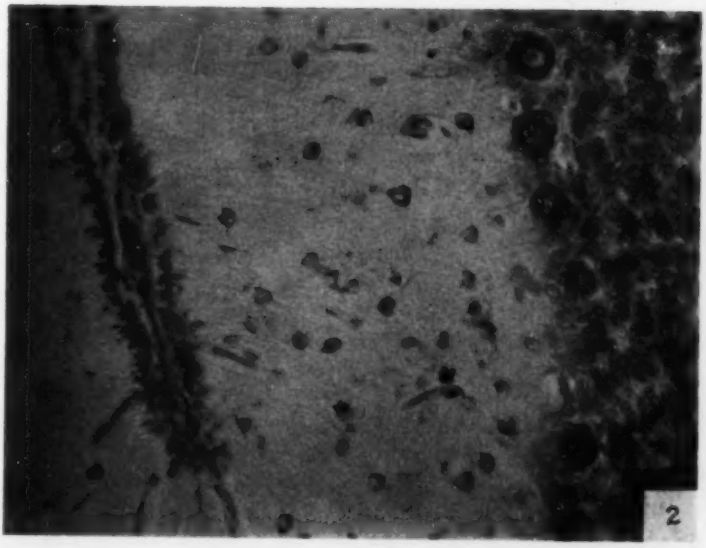
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PLATE 2

EXPLANATION OF FIGURES

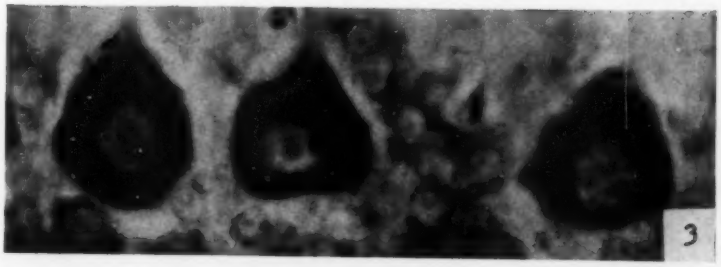
Photomicrographs of Purkinje cells from normal and magnesium deficient chicks, × 1100.

3 From chick no. 2604 fed the commercial-type control ration. The cells are of normal size. The Nissl granules are normal in appearance and have a normal distribution within the cell. Stain, toluidin blue.

4 From chick no. 2446 fed the magnesium deficient diet 3A. The cells are swollen. The Nissl granules show chromatolysis and have an abnormal distribution within the cells. Stain, toluidin blue.

5 Normal cells seen in chick no. 2604 fed the commercial-type control ration. The nuclear material has a normal appearance. Stain, hematoxylin and eosin.

6 Abnormal cells seen in chick no. 2446 fed the magnesium deficient diet 3A. The cells are swollen. The nuclear material has lost its connection with the nuclear membrane. Stain, hematoxylin and eosin.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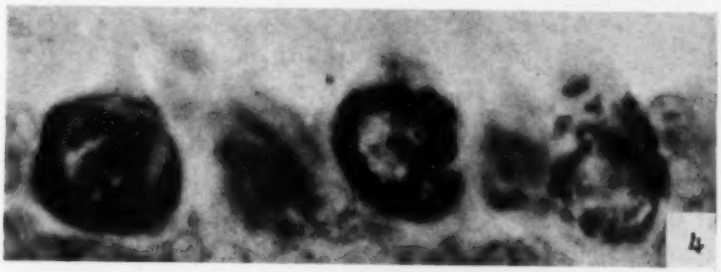
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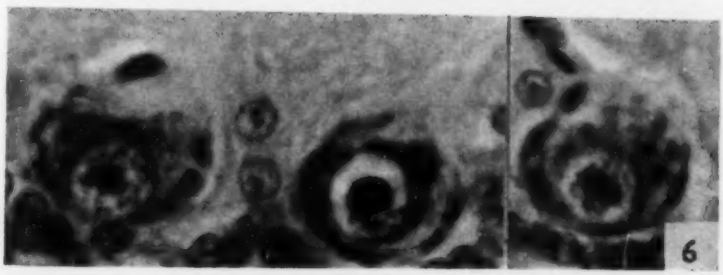
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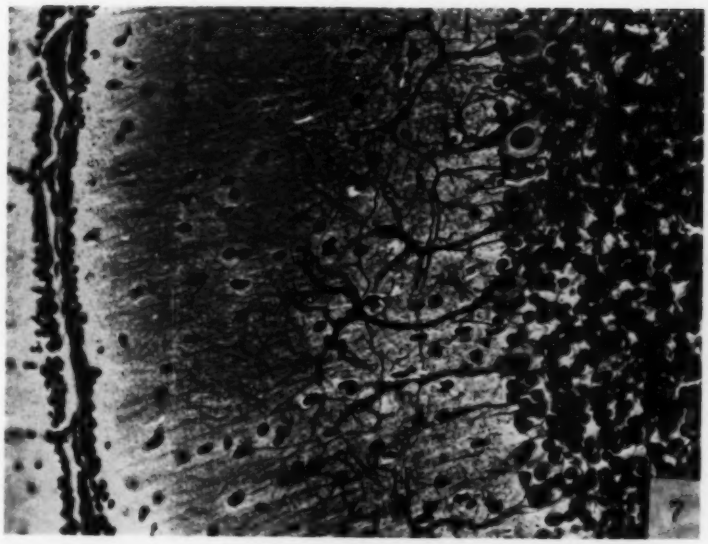
PLATE 3

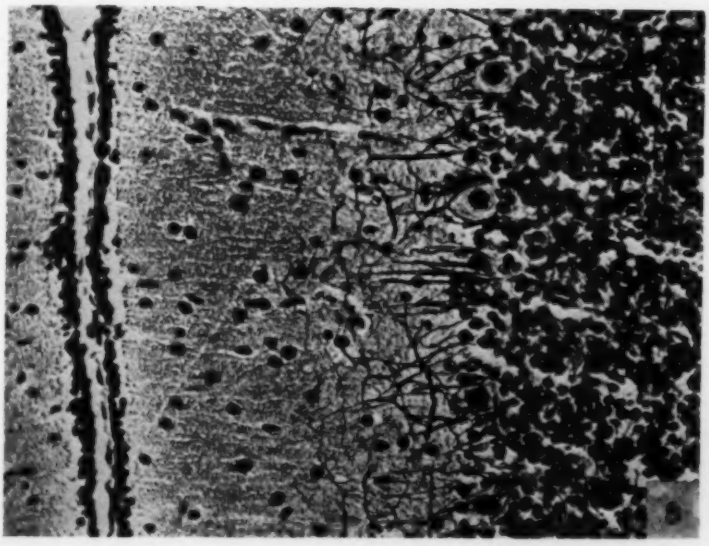
EXPLANATION OF FIGURES

Photomicrographs of parasagittal sections cut from paraffin embedded chick cerebellums stained by the Bodian protargol impregnation technique, × 315.

7 From chick no. 3620 fed the commercial-type control ration. The Purkinje cells are of normal size and their dendrites and nuclei show normal staining characteristics.

8 From chick no. 3579 fed the magnesium deficient diet 3A. The Purkinje cells are swollen and their dendrites fail to stain. The Purkinje cell's nuclei contain light staining areas and the nuclear material appears to be separated from the nuclear membrane.





A PHYSIOLOGICAL AND CYTOCHEMICAL STUDY OF THE KIDNEY AND THE ADRENAL CORTEX DURING ACUTE CHOLINE DEFICIENCY IN WEANLING RATS [1]

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TWENTY-ONE FIGURES

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The occurrence of renal lesions in weanling rats fed a diet deficient in choline was first reported by Griffith and Wade ('39). Degeneration of the kidney tubules associated with cortical hemorrhage developed in these animals after a few days on a deficient diet and could be prevented by amounts of choline too small to influence the deposition of fat in the liver. Spontaneous repair of the kidney damage occurred, furthermore, without the addition of choline to the diet if the animals survived the period of severe uremia (Griffith, '40). Griffith and Wade noted that the renal lesion was accompanied by acute involution of the thymus and suggested that this phenomenon could be the result of a generalized adaptive reaction to stress (the "alarm reaction" of Selye). In the present paper studies are reported on the interrelationships of the kidney and adrenal cortex during the course of acute choline deficiency in weanling rats.

[1] This work was supported in part by grants-in-aid to the Department of Nutrition from the Nutrition Foundation, Inc., New York, and the Milbank Memorial Fund, Inc., New York, and to the Department of Anatomy from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

In the rat it appears that the zona fasciculata of the adrenal cortex secretes hormones of the corticosterone type (C_{11}-oxy steroids concerned with carbohydrate and protein metabolism) and that the zona glomerulosa secretes hormones of the desoxycorticosterone type (C_{11}-desoxy steroids concerned with salt metabolism and renal function). During the typical adaptation reaction only the fasciculata of the adrenal cortex is affected (Deane and McKibbin, '46; Deane and Shaw, '47). It became of interest, therefore, with regard to choline deficiency in young rats, to discover (1) the relative response of the two zones of the cortex to acute renal damage; (2) the time relation between changes in the adrenal cortex and the kidney as a guide to determining cause and effect; and (3) the degree of correlation between histological changes in the kidney and changes in kidney function during the development of the renal lesion.

The general plan of these experiments was to kill choline-deficient rats at intervals during the development and regression of the renal lesions. In addition, some of the deficient animals received daily injections of desoxycorticosterone or whole adrenal extract in order to reveal whether or not hormone supplements would alleviate the kidney damage. Changes in the microscopic appearance of the adrenals and kidneys; in the levels of blood non-protein nitrogen, serum sodium and potassium, urinary sodium, potassium, creatinine and glycocyamine; and in the respiration of kidney slices were determined.

MATERIAL AND METHODS

Weanling male rats of the Sprague-Dawley and Long-Evans strains, weighing between 35 and 45 gm, were used in these experiments. No significant differences in the susceptibility of the two strains to choline deficiency were observed. The rats were divided into experimental and control groups, and the deficient animals were fed the following purified diet:[2] al-

[2] Our thanks are due Merck and Co., Inc., Rahway, N. J., the Corn Industries Research Foundation, New York, and the Sheffield Farms Co., Inc., New York, for generous supplies of materials used in the diets.

cohol-extracted peanut meal 20%; casein 6%; dextrose 64.8%; salts (Hegsted et al., '41) 4%; cod liver oil 2%; and corn oil 3%. Crystalline vitamins of the B complex were added in the following amounts per 100 gm ration: thiamine 400 μg, riboflavin 800 μg, nicotinic acid 2 mg, pyridoxine 400 μg, and calcium pantothenate 2 mg. The control rats were fed the same diet with choline chloride added at a level of 0.3%. This diet supplies 0.3% methionine, 0.5% cystine, 0.2% total organic sulfur, 0.26% sodium and 0.72% potassium. Growth rates and food consumption were recorded in all experiments. In experiments 2 and 3 (see below) the rats were maintained in metabolism cages with outside feeders and water bottles, and continuous urine collections were made. The urinals were 9-inch evaporating dishes which were screened to separate feces from the urine.

Three experiments were performed. In experiment 1, which lasted 13 days, 24 deficient and 10 control rats were used. Half of the deficient and half of the control rats were given subcutaneous injections of 1 cm^3 of aqueous beef adrenal extract [3] in two divided doses each day. This quantity of hormone will sustain growth in immature adrenalectomized rats (Olson et al., '44). Animals were killed every other day. Experiment 2 lasted 13 days also. Twenty-four rats were used, of which 16 received the deficient diet and 8 the control diet. Half of the experimental and half of the control animals received 0.5 mg desoxycorticosterone acetate in oil once daily. This amount of hormone will more than sustain normal growth in immature adrenalectomized rats (Olson et al., '44). Animals were killed at two-day intervals. In experiment 3 the rats were given no hormone treatment. Eighteen rats were started and 9 were killed — two from the deficient and one from the control groups at 18, 42 and 88 days, respectively, after the beginning of the experiment.

The animals were killed by decapitation and the blood was collected in a tube containing oxalate as an anticoagulant.

[3] We are indebted to Dr. Dwight J. Ingle of the Upjohn Co., Kalamazoo, Mich., for supplying us with the adrenal cortical extract.

Body weight and the weights of the two kidneys, the thymus, and the two adrenals were recorded.

The following chemical procedures were employed. Liver fat (total chloroform-soluble lipid) was determined by the method of Channon, Platt and Smith ('37); blood non-protein nitrogen by the method of Folin and Wu ('19); serum sodium and potassium on the Perkin-Elmer flame photometer (model 52) with an internal lithium standard;[4] urinary sodium and potassium on the flame photometer and in some experiments sodium according to the chemical procedure of Butler and Tuthill ('31); urinary creatinine by the alkaline picrate method of Lambert ('45); and urinary glycocyamine by a modification of the procedure of Dubnoff and Borsook ('41).

In experiment 1 the oxygen consumption of kidney slices was measured by the direct method of Warburg, with KOH and filter paper in the center well. The whole kidney was chilled immediately after killing the animal. Slices of kidney cortex, 0.5 mm thick, were cut and placed in chilled, oxygenated phosphate-saline of the following composition: NaCl 0.135 M, KCl 0.004 M, $CaCl_2$ 0.001 M, $MgCl_2$ 0.005 M, Na_2HPO_4: NaH_2PO_4 0.0075 M, and glucose 0.2%. The pH of this medium was 7.4. The slices were then transferred to Warburg flasks containing 3.0 cm^3 of this same medium, and the contents were equilibrated with O_2. The cocks then were closed and oxygen consumption was measured for one hour at 37°C. The dry weight of the tissue was determined after desiccation at 110°C. for two hours. The rate of oxygen consumption has been expressed as Q_{O_2}, i.e., the number of microliters of oxygen consumed per milligram of dry weight of tissue per hour.

For microscopic study, the adrenal glands and transverse slices of kidney were fixed in 10% neutralized aqueous formalin. Other slices of kidney were placed in a Zenker-formalin mixture. In a few instances slices of kidney were also fixed in formol-calcium and in "weak" Bouin's fluid for the identification of phospholipids (Baker, '46).

[4] Lithium standard: 15 mEq/L of lithium in diluted samples containing 0–1 mEq/L of sodium or potassium.

The blocks fixed in formalin were washed in running tap water for one hour, sectioned on the freezing microtome at 15 μ and prepared by a group of methods used for characterizing lipid substances (Deane, Shaw and Greep, '48; Dempsey, '48). Lipid droplets in the adrenal cortex displayed all of the reactions characteristic of droplets in the steroid-producing glands, i.e., sudanophilia, a positive Schiff reaction without pre-treatment with mercuric chloride, birefringence and a greenish-white autofluorescence. Those occurring in the kidney were sudanophilic only and were therefore judged to be triglycerides.

The slices of kidney fixed in Zenker-formalin were postchromated and embedded in paraffin; sections were cut at 3 μ and stained for 48 hours with Mallory's phosphotungstic acid hematoxylin for mitochondria. In addition, deparaffinized sections of some of the most damaged kidneys were examined under the fluorescence microscope.

Sections of kidney fixed in formol-calcium were postchromated and stained with acid hematein according to the method of Baker ('46). The control preparations, fixed in "weak" Bouin's fluid, were extracted with pyridine before mordanting and staining. Those substances staining in the first preparation but not in the second are considered to be phospholipids. The most conspicuous structures presenting this reaction in kidney are mitochondria.

RESULTS

Acute choline deficiency, with fatty liver and enlargement and congestion of the kidneys, occurred in all of the experimental animals fed the deficient diet for 5 days or more. Approximately 15% of the experimental animals died during the acute phase of the renal disease. Choline-deficient rats given adrenal hormones (either desoxycorticosterone acetate or whole adrenal extract) showed no detectable differences from untreated deficient rats, and consequently the data for all experimental animals have been pooled for the following descriptions.

Gross and chemical changes in acute choline deficiency

Although many of the renal changes in choline deficiency have been described by other investigators, a summary of our findings is submitted for the purpose of relating them to the concomitant changes in the adrenal cortex. The kidneys of the deficient rats began to enlarge on the 4th or 5th day and

TABLE 1

Changes in kidney size, the respiration of slices of kidney cortex and the gross appearance of the kidney in weanling rats fed a choline-deficient diet as compared to their controls (exp. 1)

DAYS ON DIET	NO. RATS	MEAN BODY WT.	KIDNEY WT., % BODY WT.	KIDNEY Q_{O_2} GLUCOSE 11.1 mM/L	RENAL CONGESTION [1]	RENAL ISCHEMIA [1]
		gm.				
0	2	33	1.16	19.4	0	0
3	3	52	1.18	20.5	0	0
5	3	52	1.40	18.7	2+	0
6	3	62	1.55	16.4	2+	0
7	3	51	2.10	11.0	4+	0
9	4	47	2.77	8.6	4+	1+ [2]
11	2	63	1.96	10.0	1+	2+ [2]
12	2	70	1.63	13.8	0	3+
13	3	72	1.16	13.3	0	2+
11–13 [3]	3	97	1.05	18.1	0	0

[1] The degree of congestion and ischemia was determined by gross inspection of the kidneys.

[2] Indicates that pale areas occurred on the surface of the congested kidney.

[3] This group was fed the control purified diet during the experimental period.

attained a maximum size on the 9th day of 2.74% of body weight (range: 2.24–3.19%). Congestion of the renal cortex and subscapular hemorrhage appeared on the 5th day and increased *pari passu* with kidney enlargement (table 1). After the 9th day the kidneys began to decrease in relative size and became pale and ischemic. Proportional kidney weight was restored nearly to normal in the deficient animals after 13 days of deficiency.

With the onset of renal damage, food consumption declined and the animals lost weight (fig. 1). The weight of the paired adrenal glands increased from an initial 31.2 mg to 46.6 mg per 100 gm rat body weight on the 9th day. (The absolute weights of the adrenals rose from 15.6 mg at zero days to 23.4 mg at 9 days.) Marked thymus atrophy coincided with the greatest enlargement of the adrenals. After the 9th day the weights of both the thymus and the adrenals returned toward normal and by the 18th day had attained sizes proportionately comparable to those of the control rats. Liver fat, initially 4.1% of the fresh liver weight, rose to 21.1% at 5 days, declined to 12.8% on the 11th day, and returned to 21.7% on the 13th day. The transient decline of liver fat during the period of acute renal damage was probably due to the inanition which supervened at this time.

Both water consumption and urine production decreased during the phase of renal damage. The excretion of sodium and potassium reached a minimum at 9 to 11 days (table 2; fig. 2); at the same time the serum levels were increased—sodium from 149 to 155 mEq/L and potassium from 6.3 to 9.0 mEq/L. The excretion of creatinine (expressed in terms of milligrams of creatinine excreted per kilogram of rat per day) declined 35% during the period of acute renal degeneration. The excretion of glycocyamine, on the other hand, showed a phasic variation. As is shown in table 2, the glycocyamine coefficient (milligrams of glycocyamine excreted per kilogram of rat per day) increased in the early stages of the deficiency (zero to 4 days) but declined sharply to about half that of normal during the period of renal failure. Blood non-protein nitrogen began to increase at the onset of enlargement of the kidney and reached its highest value of 285 mg% on the 11th day (fig. 2). All of these indices of renal function had returned essentially to normal by the 13th day of deficiency.

The respiration of slices of kidney cortex *in vitro* remained normal during the first 5 days of the deficiency (table 1). Thereafter there was a marked decrease in oxygen consumption, the Qo_2 declining from a control value of about 19 to

8.6 on the 9th day. During the next few days the oxygen consumption increased but was still significantly below normal on the 13th day when the experiment was terminated.

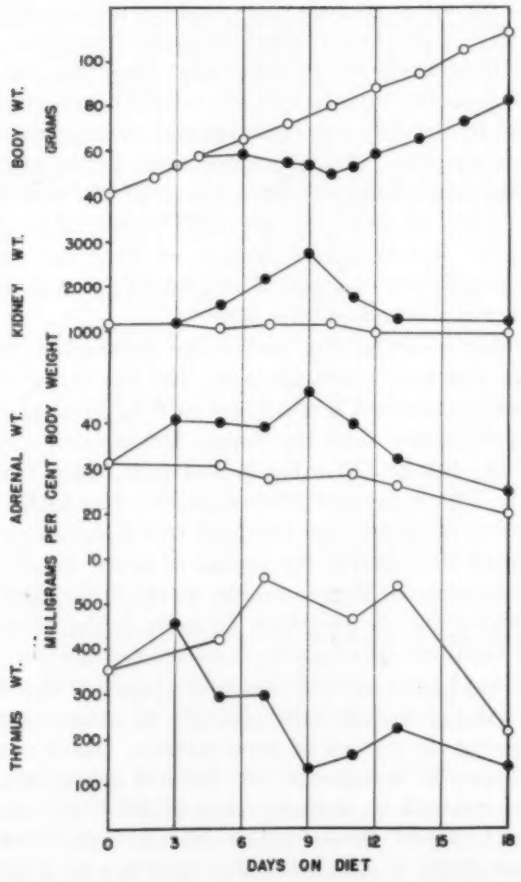

Fig. 1 Changes in body weight and proportional size of the kidneys, adrenals and thymus of weanling rats fed a low choline diet and of their controls. Values for deficient animals, solid circles; values for controls, open circles. Each point represents average data, compiled from all three experiments, for two to 10 rats.

TABLE 2

Urinary excretion of sodium, potassium, creatinine and glycocyamine by choline-deficient and control rats at two stages in the experimental period (exp. 2)

URINARY CONSTITUENT	NO. RATS		VALUES IN MG PER KG RAT PER DAY			
			0–4 DAYS		9–11 DAYS	
	Control	Deficient	Control	Deficient	Control	Deficient
Sodium	10	16	247	258	254	125
Potassium	10	16	988	937	853	398
Creatinine	10	20	36.0	35.8	36.2	23.5
Glycocyamine	10	20	14.4	27.6	14.1	7.9

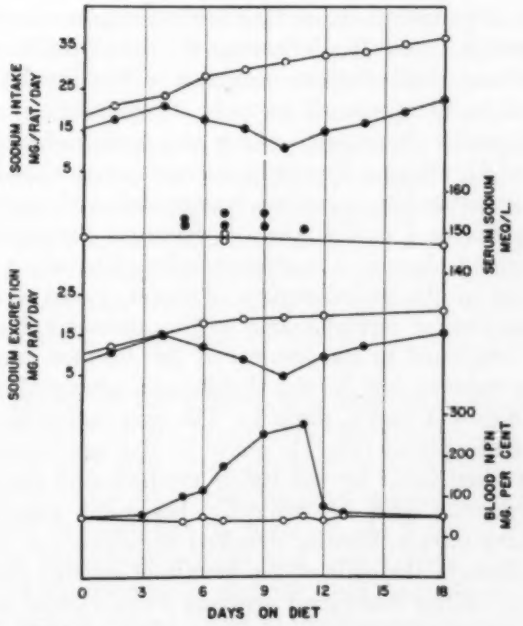

Fig. 2 Changes in sodium intake, serum sodium, sodium excretion, and blood non-protein nitrogen in weanling rats fed a low choline diet and in their controls. Values for deficient animals, solid circles; values for controls, open circles. With the exception of the serum sodium values, which are individual rats, each point represents average data taken from experiments 2 and 3 for two to 10 rats.

Cytological changes in kidney and adrenal in acute choline deficiency

Kidney. Between the third and 5th days small, infranuclear droplets of fat appeared in the tubular cells of the kidney; these were most common in the proximal segments but occurred also in some distal segments. The amount of fat increased rapidly until the 9th day, with both the enlargement of droplets and the involvement of new tubules (figs. 8, 10 and 11, plates 1 and 2). Between the 5th and 7th days congestion of blood appeared in the peritubular plexuses; Hartroft ('48) has demonstrated that this congestion results from blood stasis. By the 9th day hemorrhage beneath the capsule and stasis throughout the cortex were conspicuous (figs. 4, 9 and 11, plates 1 and 2). Following the development of blood stasis, tubular degeneration occurred in the involved areas. Mitochondria disappeared entirely from tubular cells immediately under the capsule where hemorrhage had occurred (figs. 4 and 9), whereas deeper in the cortex mitochondria were still recognizable but appeared fragmented and sparse, as is shown in figures 7 and 8, plate 1. Sections prepared by the Baker method showed a comparable fragmentation and disappearance of the mitochondria. Figures 3 and 6 illustrate the appearance of mitochondria in the normal kidney.

Casts developed in the lumens of the tubules, not only in collecting tubules but in the distal and proximal segments as well (figs. 4, 7 and 8, plate 1). The cast material was only faintly sudanophilic (fig. 11, plate 2), did not contain phospholipids identifiable by the Baker method, and stained with phosphotungstic acid hematoxylin, the color ranging from pink to dark purple. Similar droplets were present in the apical cytoplasm of the cells of the proximal tubules (figs. 8 and 9, plate 1). When unstained sections were viewed under the fluorescence microscope, the casts and intracellular droplets emitted a deep reddish-brown fluorescence similar to that of the static blood; when stained with acid hematein after extraction with pyridine, the cast material was blackened in a fashion identical to the erythrocytes. It seems probable, there-

fore, that the cast material consisted in part of hemoglobin or hemoglobin derivatives.

After the 9th day, the kidney circulation was restored quite suddenly, and the static and extravasated blood promptly disappeared. By the 13th day all cast material had been removed and fat had diminished in all but a few necrotic tubules immediately below the capsule (fig. 12, plate 2). The tubules near the medulla appeared virtually normal but those in the peripheral third of the cortex continued to lack demonstrable mitochondria (fig. 5, plate 1). The microscopic appearance of the kidney was not studied beyond the 13th day of deficiency.

Adrenal cortex. The adrenal cortices of rats fed the complete purified diet appeared entirely normal (figs. 13 and 16, plates 2 and 3). In deficient animals the cortex began to broaden on the 5th day and continued to enlarge concomitantly with the increase in kidney weight. This broadening and the increase in adrenal weight mentioned earlier reflected principally a thickening of the fasciculata (figs. 18 and 19, plate 3), although the glomerulosa also became wider. As the fasciculata hypertrophied, the lipid droplets within its cells became small and the birefringent particles uniformly fine (fig. 14, plate 2). This zone returned to normal width on the 11th day.

The changes in the glomerulosa were even more remarkable than those of the fasciculata. By the 5th day the lipid droplets in this zone were greatly reduced in number (fig. 17, plate 3). At 7 days the droplets appearing in this zone were small in size (fig. 18, plate 3). At 9 days, the time of maximum renal enlargement and hemorrhage, the zone had broadened conspicuously and its lipid had all but vanished (figs. 14 and 19, plates 2 and 3). Subsequently small droplets returned (figs. 15 and 20, plates 2 and 3). although the zone remained somewhat thickened through the 18th day. Both the glomerulosa and fasciculata were normal in appearance by the 42nd day of deficiency (fig. 21, plate 3).

DISCUSSION

Kidney damage

The pathogenesis of the hemorrhagic kidney in acute choline deficiency in young rats is not entirely clear, despite the contributions of several investigators (György and Goldblatt, '40; Engel and Salmon, '41; Christensen, '42; Hartroft, '47, '48; and Dessau and Oleson, '47). In reviewing the development of the kidney lesion, it is our purpose to correlate the cytological changes observed by us and by others with changes in renal function and simultaneous changes in the adrenal cortex. All investigators agree that the initial lesion in the kidney cortex is stasis caused by blockage of the capillaries around the tubules rather than by any change in the glomeruli. According to Hartroft, this blockade is caused by fatty infiltration of the tubular cells, in consequence of which they swell and occlude the vessels of the peritubular plexuses. Our observations are entirely compatible with this explanation. Since Patterson, Keevil and McHenry ('44) found a decrease in phosphatide turnover, as measured with P_{32}, in the kidneys from young choline-deficient rats, the fatty infiltration may result from this decrease in turnover.

Secondary pathological changes develop after stasis and the resultant anoxia have occurred, as evidenced both by the decrease in oxygen consumption of kidney slices *in vitro* and by the fragmentation and even disappearance of mitochondria in the tubular cells. Under conditions of anoxia, fragmentation and dissolution of mitochondria have previously been observed both in kidney and liver cells (Emmel, '40; Deane, Nesbett, Buchanan and Hastings, '47). Furthermore, the hemorrhage and stasis apparently result in hemolysis and the passage of hemoglobin into the lumens of the tubules, where it forms casts. A similar mechanism for cast formation has been described in other experimental situations by Monke and Yuile ('40) and Oliver ('44). Droplets of cast material in the supranuclear cytoplasm of proximal tubule cells are believed indicative of resorption (Gérard, '36; Smetana and Johnson, '42).

During the period of stasis and hemorrhage, renal function is markedly depressed, as indicated by oliguria, nitrogen retention, and a decrease in the excretion of sodium, potassium, creatinine and glycocyamine. With greatest kidney damage the creatinine excretion fell 35%. Since the rat excretes creatinine by way of the glomeruli (Friedman, '47), and since the excretion is unaffected by starvation or lack of dietary methyl groups (Olson, Eder and Stare, '47), this reduction in creatinine excretion suggests a decrease in effective filtration. A similar conclusion may be drawn from the changes in the excretion of electrolytes. The excretion of sodium and potassium was depressed by comparable amounts (about 50%), an effect unlike that induced by adrenal hormones. Moreover, in deficient rats given extra desoxycorticosterone acetate (0.5 mg/day), the excretion of sodium was not significantly changed from that of uninjected animals. In addition, Griffith ('41) found a similar decline in the excretion of phenol red, which is excreted chiefly by the tubules. All of these facts are consistent with the view that in acute choline deficiency whole nephrons are inactivated as a result of the blood stasis.

The excretion of glycocyamine, a compound synthesized by the tubules, was depressed more than was creatinine excretion during the period of maximum kidney damage. The studies of Borsook and Dubnoff ('40), Du Vigneaud et al. ('40) and Bloch and Schoenheimer ('41) indicate that glycocyamine is formed in the kidney from arginine and glycine and is then methylated to creatine in the liver. In adult rats deficient in methyl groups but without any detectable damage to the kidney, glycocyamine excretion rises, presumably because of retarded conversion to creatine in the liver (Olson, Eder and Stare, '47). A similar increase in the excretion of glycocyamine occurred in the young choline-deficient rats of the present study before the onset of kidney congestion. During the height of the renal damage, however, the excretion of glycocyamine declined markedly, most likely because of the

damage to the tubular parenchyma and the consequent failure of these cells to synthesize glycocyamine.

A decrease in the respiration of kidney slices followed the development of blood stasis by one or two days, thus paralleling other signs of reduced tubular activity. This decrease in respiratory capacity went hand in hand with fragmentation and destruction of mitochondria in the tubular cells. Even after restoration of the renal circulation and nitrogen clearance, slices of kidney cortex taken from the periphery, where mitochondrial destruction persisted, showed a depressed oxygen consumption. Mitochondria have been found to contain several important oxidative enzymes, such as cytochrome C, cytochrome oxidase, succinoxidase, and fatty acid oxidase (Lazarow, '43; Hogeboom, Claude and Hotchkiss, '46; Kennedy and Lehninger, '48). It is not unexpected, therefore, that the dissolution of mitochondria in the cells of the renal tubules is associated with a decline in oxygen consumption of slices *in vitro*. In the early phases of this work it was thought conceivable that severe lack of choline might directly interfere with the formation of mitochondria, since the latter contain considerable amounts of phospholipid (Lazarow, '43). It turned out, however, that the dissolution of mitochondria followed instead of preceding blood stasis. Nevertheless, other workers have demonstrated that choline-deficient rats display a decreased respiration in tissues not suffering from blood stasis (liver, Welch, Irving and Best, '35; striated muscle, Abdon and Borglin, '46). In addition, György ('47) has reported tubular nephrosis which was not secondary to kidney congestion in adult rats fed a choline-deficient diet.

Changes in the adrenal cortex

As regards the interrelationships of the kidney and the adrenal cortex in this syndrome, changes in adrenal weight and adrenal cytochemistry paralleled the changes in kidney size and histology during the development and resolution of the renal lesion. No abnormalities of the adrenal cortex

persisted after the recovery of the kidney, despite continued fatty infiltration of the liver. On the other hand, the administration of neither desoxycorticosterone acetate nor whole adrenal extract had any effect on the progress of the renal lesion. It seems clear, therefore, that adrenal insufficiency does not play a rôle in the etiology of the kidney damage, but that the changes in the adrenal cortex are secondary to the renal changes.

Both the zona glomerulosa and the zona fasciculata appeared hyperactive at the time of maximum renal damage, although the glomerulosa appeared far more stimulated. The enlargement of the fasciculata and reduction in the size of its lipid droplets, accompanied by marked atrophy of the thymus gland, suggest an increased secretion of 11-oxy-corticosteroids during the acute phase of the deficiency (Dougherty and White, '45; Deane and Shaw, '47; Bergner and Deane, '48). The immediate recovery of the fasciculata and the thymus upon restoration of kidney circulation and normal food intake, however, together with the relatively mild depletion of fasciculata lipids during the phase of renal damage, suggest that the changes in the fasciculata were referable to the "alarming" stimulus of general inanition and uremia. This stimulation of the fasciculata and the decline in liver fat appear to have been the only changes referable to inanition. Contrary to the conclusions of Christensen and Griffith ('42), there is no evidence of a direct effect of choline deficiency on the pituitary-adrenal-thymus apparatus as in the case of pantothenic acid and thiamine deficiencies (Deane and McKibbin, '46; Deane and Shaw, '47).

The marked hyperactivity of the zona glomerulosa during the period of renal damage, a reaction not noted in other conditions of physiological stress, is the outstanding feature of the adrenal response in acute choline deficiency of young rats. Both hypertrophy of the zone and hypersecretion, as indicated by complete disappearance of lipids (Deane, Shaw and Greep, '48), occurred at the time of maximum kidney

damage. Furthermore, recovery was somewhat slower in this zone than in the fasciculata.

The question of the mechanism by which the kidney damage stimulates secretion by the glomerulosa is of some interest. The glomerulosa, unlike the fasciculata, appears to be independent of pituitary control in the rat (Deane and Greep, '46), and it is believed to secrete salt-regulating hormones of the desoxycorticosterone type (Greep and Deane, '47; Nichols, '48; Deane, Shaw and Greep, '48). Deane, Shaw and Greep have shown that the zona glomerulosa of the rat becomes hyperactive with either sodium deficiency or potassium excess, i.e., whenever there is a decrease in the ratio of sodium to potassium in the blood plasma. During the acute phase of choline deficiency there is a fall in the ratio of sodium to potassium in the plasma, despite absolute increases in both. It seems possible, therefore, that this shift might constitute the stimulus for increased glomerulosa activity.

At the present time, of course, other causes for the stimulation of the glomerulosa cannot be ruled out. That the high level of non-protein nitrogen was not the cause is suggested by the fact that the nitrogen retention persisted after the zona glomerulosa had begun to recover (11 days). Moreover, in several rats made acutely uremic either by removal of the kidneys or ligation of the renal arteries, no changes in the glomerulosa were demonstrable. It remains possible, however, that some humoral product of the damaged kidney could stimulate the glomerulosa.

SUMMARY

The effects of acute choline deficiency in weanling male rats on the cytochemistry of the adrenal cortex and the cytology and function of the kidney have been studied.

The renal lesion of acute choline deficiency is characterized by fatty infiltration of the tubular epithelium, congestion and hemorrhage of the cortex, fragmentation and disappearance of mitochondria in the tubular epithelium, and a decrease in the respiration of slices of kidney cortex *in vitro*. At the time

of maximum kidney damage (9 days), casts of material resembling hemoglobin appear in the lumens of the tubules.

Concomitant with the renal congestion there is nitrogen and electrolyte retention. The excretion of glycocyamine is depressed more than is the excretion of creatinine.

The renal damage is accompanied by an enlargement of the adrenal cortex and atrophy of the thymus. The zona fasciculata appears only moderately stimulated, apparently as a result of the "alarming" stimulus of inanition. The zona glomerulosa is much more markedly stimulated, possibly as a result of altered electrolyte balance during renal failure.

With kidney repair, which occurs without added choline, the adrenal cortex returns to normal, the zona fasciculata recovering more rapidly than the zona glomerulosa. Both zones appear normal by the 42nd day of deficiency, despite the continued fatty liver.

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LITERATURE CITED

ABDON, N. O., AND N. E. BORGLIN 1946 Disturbances in oxidative metabolism in choline deficiency. Nature, *158:* 793.

BAKER, J. R. 1946 The histochemical recognition of lipine. Q. J. Micro. Sci., *87:* 440.

BERGNER, G. E., AND H. W. DEANE 1948 Effects of pituitary adrenocorticotropic hormone on the intact rat, with special reference to cytochemical changes in the adrenal cortex. Endocrinology, *43:* 240.

BLOCH, K., AND R. SCHOENHEIMER 1941 The biological precursors of creatine. J. Biol. Chem., *138:* 167.

BORSOOK, H., AND J. W. DUBNOFF 1940 The formation of creatine from glycocyamine in the liver. Ibid., *132:* 559.

BUTLER, A. M., AND E. TUTHILL 1931 An application of the uranyl zinc acetate method for determination of sodium in biological material. Ibid., *93:* 171.

CHANNON, H. J., A. P. PLATT AND J. A. B. SMITH 1937 The dietary prevention of fatty livers. Two analogues of choline. Biochem. J., *31:* 1736.

CHRISTENSEN, K. 1942 Renal changes in the albino rat on low choline and choline-deficient diets. Arch. Path., *34:* 633.

CHRISTENSEN, K., AND W. H. GRIFFITH 1942 Involution and regeneration of thymus in rats fed choline-deficient diets. Endocrinology, *30:* 574.

DEANE, H. W., AND R. O. GREEP 1946 A morphological and histochemical study of the rat's adrenal cortex after hypophysectomy, with comments on the liver. Am. J. Anat., *79:* 117.

DEANE, H. W., AND J. M. MCKIBBIN 1946 The chemical cytology of the rat's adrenal cortex in pantothenic acid deficiency. Endocrinology, *38:* 385.

DEANE, H. W., F. B. NESBETT, J. M. BUCHANAN AND A. B. HASTINGS 1947 A cytochemical study of glycogen synthesized from glucose or pyruvate by liver slices *in vitro*. J. Cell. and Comp. Physiol., *30:* 255.

DEANE, H. W., AND J. H. SHAW 1947 A cytochemical study of the responses of the adrenal cortex of the rat to thiamine, riboflavin and pyridoxine deficiencies. J. Nutrition, *34:* 1.

DEANE, H. W., J. H. SHAW AND R. O. GREEP 1948 The effect of altered sodium and potassium intake on the width and cytochemistry of the zona glomerulosa of the rat's adrenal cortex. Endocrinology, *43:* 133.

DEMPSEY, E. W. 1948 The chemical cytology of endocrine glands. Recent Progress in Hormone Research, *3:* 127.

DESSAU, F. I., AND J. J. OLESON 1947 Nature of renal changes in acute choline deficiency. Proc. Soc. Exp. Biol. Med., *64:* 278.

DOUGHERTY, T. F., AND A. WHITE 1945 Functional alterations in lymphoid tissue induced by adrenal cortical secretion. Am. J. Anat., *77:* 81.

DUBNOFF, J. W., AND H. BORSOOK 1941 A micro method for the determination of glycocyamine in biological fluids and tissue extracts. J. Biol. Chem., *138:* 381.

DU VIGNEAUD, V., J. P. CHANDLER, M. COHN AND G. B. BROWN 1940 The transfer of the methyl group from methionine to choline and creatine. Ibid., *134:* 787.

EMMEL, V. M. 1940 Mitochondrial and pH changes in the rat's kidney following interruption and restoration of the renal circulation. Anat. Rec., *78:* 361.

ENGEL, R. W., AND W. D. SALMON 1941 Improved diets for nutritional and pathologic studies of choline deficiency in young rats. J. Nutrition, *22:* 109.

FOLIN, O., AND H. WU 1919 A system of blood analysis. J. Biol. Chem., *38:* 81.

FRIEDMAN, M. 1947 The creatinine, insulin and hippurate clearance in the rat. Am. J. Physiol., *148:* 387.

GÉRARD, P. 1936 Comparative histophysiology of the vertebrate nephron. J. Anat., *70:* 354.

GREEP, R. O., AND H. W. DEANE 1947 Cytochemical evidence for the cessation of hormone production in the zona glomerulosa of the rat's adrenal cortex after prolonged treatment with desoxycorticosterone acetate. Endocrinology, *40:* 417.

GRIFFITH, W. H. 1940 Choline metabolism. IV. The relation of age, weight and sex of young rats to the occurrence of hemorrhagic degeneration on a low choline diet. J. Nutrition, *19:* 437.

———— 1941 The relation of choline to the kidneys. Biol. Sympos., *5:* 193.

GRIFFITH, W. H., AND N. J. WADE 1939 Choline metabolism. I. The occurrence and prevention of hemorrhagic degeneration in young rats on a low choline diet. J. Biol. Chem., *131:* 567.

GYÖRGY, P. 1947 Discussion of paper by Hartroft ('47), Sixth Conference on Liver Injury (Josiah Macy, Jr., Found.): 49.

GYÖRGY, P., AND H. GOLDBLATT 1940 Choline as a member of the vitamin B_2 complex. J. Exp. Med., *72:* 1.

HARTROFT, W. S. 1947 Some histological aspects of renal and hepatic acholinopathy. Sixth Conference on Liver Injury (Josiah Macy, Jr., Found.): 41.

———— 1948 Pathogenesis of renal lesions in weanling and young adult rats fed choline-deficient diets. Brit. J. Exp. Path., *29:* 483.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.

HOGEBOOM, G. H., A. CLAUDE AND R. D. HOTCHKISS 1946 The distribution of cytochrome oxidase and succinoxidase in the cytoplasm of the mammalian liver cell. Ibid., *165:* 615.

KENNEDY, E. P., AND A. L. LEHNINGER 1948 Intracellular structures and the fatty acid oxidase system of rat liver. Ibid., *172:* 847.

LAMBERT, G. F. 1945 The determination of creatine and creatinine. Ibid., *161:* 679.

LAZAROW, A. 1943 The chemical structure of cytoplasm as investigated in Professor Bensley's laboratory during the past 10 years. Biol. Sympos., *10:* 9.

MONKE, J. V., AND C. L. YUILE 1940 The renal clearance of hemoglobin in the dog. J. Exp. Med., *72:* 149.

NICHOLS, J. 1948 Effects of electrolyte imbalance on the adrenal gland. Arch. Path., *45:* 717.

OLIVER, J. 1944 New directions in renal morphology: A method, its results and its future. Harvey Lectures, *40:* 102.

OLSON, R. E., H. A. EDER AND F. J. STARE 1947 Excretion of glycocyamine by rats on low methionine-low choline diets. Fed. Proc., *6:* 283.

OLSON, R. E., F. A. JACOBS, D. RICHERT, S. A. THAYER, L. J. KOPP AND N. J. WADE 1944 The comparative bioassay of several extracts of the adrenal cortex in tests employing four separate physiological responses. Endocrinology, *35:* 430.

PATTERSON, J. M., N. B. KEEVIL AND E. W. MCHENRY 1944 Choline and the prevention of hemorrhagic kidneys in the rat. II. Phospholipid turnover determined with radioactive phosphorus. J. Biol. Chem., *153:* 489.

SMETANA, H., AND F. R. JOHNSON 1942 The origin of colloid and lipoid droplets in the epithelial cells of the renal tubules. Am. J. Path., *18:* 1029.

WELCH, M. S., L. IRVING AND C. A. BEST 1935 The effect of choline on the oxygen consumption of normal and fatty livers of the rat. Am. J. Physiol., *113:* 136 (Proc.).

PLATE 1

EXPLANATION OF FIGURES

All figures on this plate are from kidneys fixed in Zenker-formalin solution and postchromated. Paraffin sections were cut at 3 μ and stained with Mallory's phosphotungstic acid hematoxylin for 48 hours. Figures 3 to 5, photomicrographs taken with combined green and yellow filters, $\times$ 45. The preparations were stained and photographed indentically. Figures 6 to 9, drawings made with the aid of a camera lucida, $\times$ 90 objective, $\times$ 10 ocular; $\times$ 1690 at the plane of the drawing.

3 Normal kidney, showing cortex and part of the medulla. The proximal convoluted tubules stain darkly, principally because of their many, highly chromophilic mitochondria (fig. 6). The distal convoluted tubules stain more lightly, and the collecting tubules and Henle's loops only faintly.

4 Kidney of a rat fed the choline-deficient diet for 9 days. Underneath the capsule hemorrhage (H) has occurred; a considerable amount of stagnant blood (S) is visible between the tubules in the outer half of the cortex. Here the tubules no longer stain normally, whereas they stain almost normally in the inner part of the cortex (compare figs. 7, 8 and 9). Many tubules contain homogeneous chromophilic casts: generally these stain purple, sometimes pink. The glomeruli appear normal.

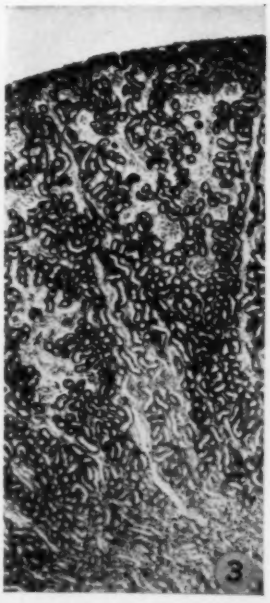
5 Kidney of a rat fed the choline-deficient diet for 13 days. All extravasated and stagnant blood has disappeared, as have the casts. Immediately underneath the capsule the tubules appear collapsed and mitochondria have not reappeared. In the inner part of the cortex the tubules stain in a nearly normal fashion.

6 Normal kidney showing a proximal tubule on the left and a distal tubule on the right. In the cells of the proximal tubule, the long filamentous mitochondria are oriented perpendicularly to the basement membrane and the cytoplasm is quite chromophilic. In the distal tubule the mitochondria are sparser and less well oriented.

7 Kidney of a rat fed the choline-deficient diet for 9 days. Deep portion of cortex, illustrating a proximal tubule on the left and a distal one on the right. Stagnant blood within the capillaries. The mitochondria in the proximal tubule are sparser than normal, and the cytoplasm is less chromophilic. The mitochondria in the distal tubule stain only faintly. A cast is present in lumen of the distal tubule.

8 Kidney of a rat fed the choline-deficient diet for 9 days. Mid-portion of cortex, showing a collecting tubule on the left, and a proximal tubule on the right. Casts are present in the lumens of both. The cells of the proximal tubule contain unstained fatty spaces with a few, light-staining mitochondrial fragments between them. The larger chromophilic spherules in these cells stain in the same fashion as the cast material.

9 Kidney of a rat fed the choline-deficient diet for 9 days. Subcapsular region of cortex, illustrating a proximal tubule surrounded by stagnant blood. The tubule appears collapsed and the cells completely distorted by large chromophilic inclusion bodies. The faintly staining granules within their cytoplasm may be remnants of mitochondria.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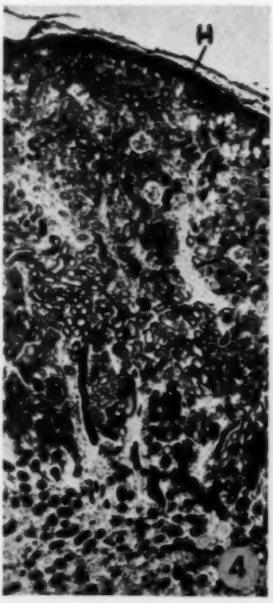
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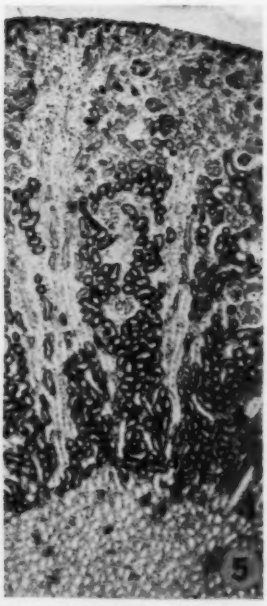
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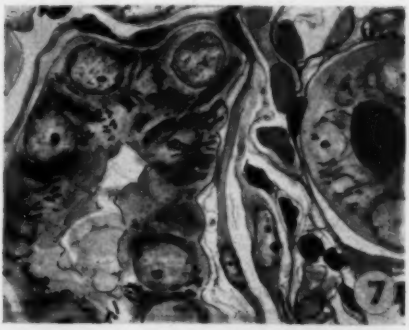
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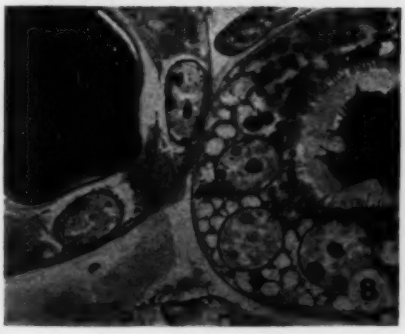
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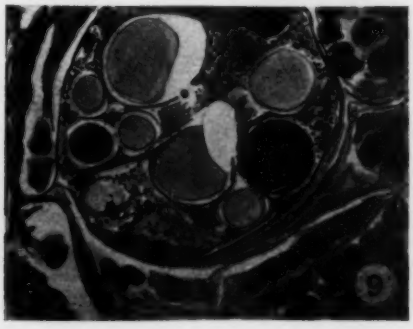
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PLATE 2

EXPLANATION OF FIGURES

All figures on this plate are photomicrographs of organs fixed in 10% formalin for at least two days and then sectioned at 15 μ on the freezing microtome, $\times$ 90. Figures 10 to 12 are of kidneys, stained with Sudan black B for fatty substances, and photographed with a blue filter. Figures 13 to 15 are of adrenals, photographed under the birefringence microscope; the margin of the capsule has been drawn in by hand.

10 Kidney of a rat fed the choline-deficient diet for 7 days. Droplets of fat (F) stained black with the Sudan dye occur at the bases of the cells in many of the convoluted tubules.

11 Kidney of a rat fed the choline-deficient diet for 9 days. Considerable hemorrhage (H) is visible beneath the capsule. Fat droplets occur in most of the tubules, being particularly concentrated in isolated groups of subcapsular tubules. Gray-staining casts (C) are visible in many tubules. The increase in fat described here was clearly seen in the original material under greater magnification.

12 Kidney of a rat fed the choline-deficient diet for 13 days. Little fat is present except in occasional peripheral tubules. The gray staining of the inner tubules is indicative of mitochondria. The epithelium of some of the outer tubules has collapsed or been sloughed off.

13 Adrenal cortex of a rat fed the control diet for 9 days. Birefringent material characterizes the narrow glomerulosa and the fasciculata. The particles vary in size, some being fine and some coarse.

14 Adrenal cortex of a rat fed the choline-deficient diet for 9 days. The glomerulosa appears broadened. Only occasional, fine birefringent particles are present. The birefringent material in the fasciculata is uniformly fine and is limited to the outer half of the zone.

15 Adrenal cortex of a rat fed the choline-deficient diet for 13 days. The glomerulosa is broad but contains considerable birefringent material, mostly fine. The fasciculata is crowded with birefringent particules, many coarser than in figure 14.

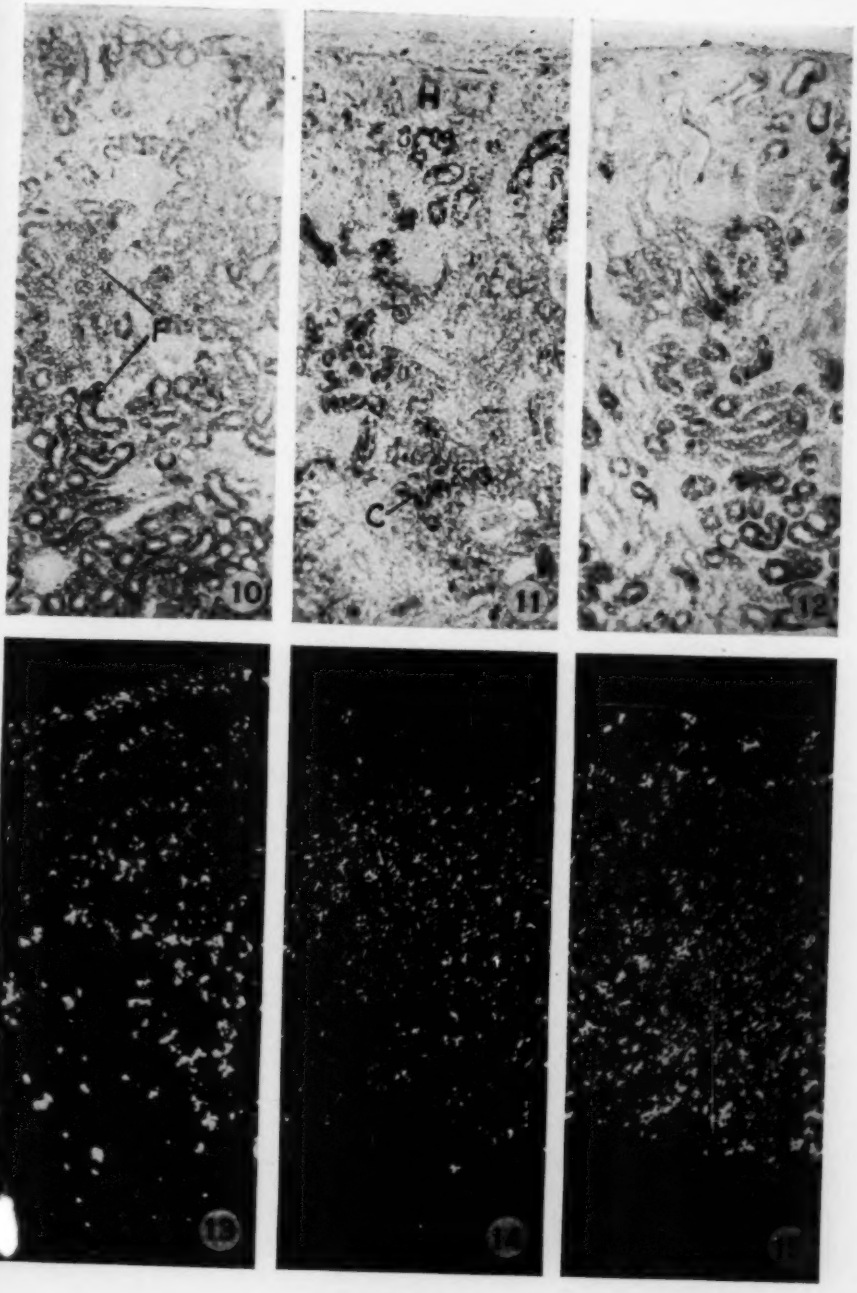
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PLATE 3

EXPLANATION OF FIGURES

All photomicrographs on this plate are of adrenal glands that were fixed in 10% formalin for at least two days, then sectioned on the freezing microtome at 15 μ. Figures 16 and 21 are from sections stained with Sudan IV and photographed with a blue filter. Figures 17 to 20 are from sections stained with Schiff's reagent and photographed with green and yellow filters combined. All × 90.

16 Adrenal cortex of a rat fed the control purified diet for 10 days. The cortex appears normal for a rat weighing 55 gm. Considerable lipid is present in the narrow zona glomerulosa, none in the transitional zone, and a great deal throughout the fasciculata. No zona reticularis occurs in the cortex of the weanling rat.

17 Adrenal cortex of a rat fed the choline-deficient diet for 5 days. Schiff-positive lipid droplets are virtually absent from the glomerulosa. The fasciculata is slightly broadened and its lipid material reacts intensely with the Schiff reagent.

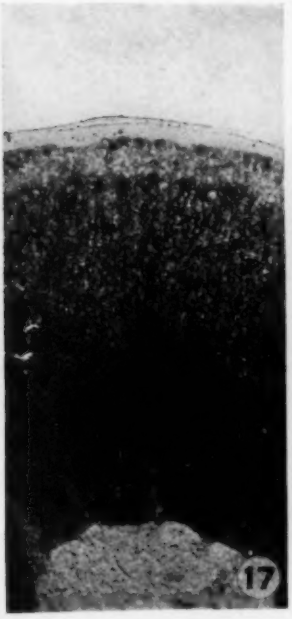
18 Adrenal cortex of a rat fed the choline-deficient diet for 7 days. The lipid droplets in both the glomerulosa and fasciculata are Schiff-positive. The fasciculata is further broadened.

19 Adrenal cortex of a rat fed the choline-deficient diet for 9 days. The glomerulosa appears broad and completely depleted of Schiff-positive material. The fasciculata is extremely broad but not depleted.

20 Adrenal cortex of a rat fed the choline-deficient diet for 13 days. The glomerulosa remains broad but contains considerable amounts of Schiff-positive lipid. The fasciculata has shrunk to normal size.

21 Adrenal cortex of a rat fed the choline-deficient diet for 42 days. This gland appears normal for a rat weighting 175 gm. Less lipid is present in the inner fasciculata than in the younger rat (fig. 16).

16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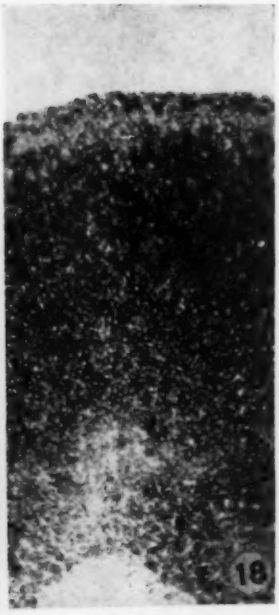
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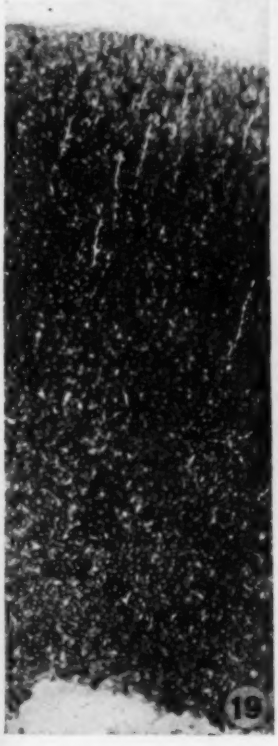
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NITROGEN EXCRETION OF WOMEN RELATED TO THE DISTRIBUTION OF ANIMAL PROTEIN IN DAILY MEALS[1]

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Research with animals has shown the inability of the body to store amino acids for use at a later time, and has demonstrated that the body needs to have all the essential amino acids present simultaneously and in adequate amounts for tissue synthesis (Geiger, '47; Cannon et al., '47; Schaeffer and Geiger, '47; Harte, Travers and Sarich, '48). Henry and Kon ('46) found that the supplementary relationship of milk and potato, and of bread and cheese, did not exist if these foods were fed separately on alternate days but could be demonstrated only if the two foods were fed together. Geiger ('48) has shown that diets containing wheat gluten + blood protein, or yeast + blood protein, or yeast + wheat gluten protein promote growth satisfactorily. Growth did not result, however, when these same pairs of proteins were fed separately during alternating 10-hour periods with two-hour intervals between feedings.

The possibility warrants study that the nitrogen metabolism of human subjects may be influenced by the time of in-

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gestion of proteins, especially from the standpoint of the distribution of complete proteins among the usual daily meals. If one meal, such as breakfast, is completely devoid of a high quality complete protein, there is the possibility that the nitrogen utilization of the individual will be impaired even though the total daily intake of protein is adequate.

The study reported here is a preliminary investigation of whether the time element in the feeding of complete animal protein affects the utilization of nitrogen by human subjects. It seemed pertinent to compare under controlled conditions (a) the daily nitrogen metabolism when the amount of protein in the diet was adequate but no animal protein was included in the breakfast, and (b) the nitrogen metabolism on the same protein intake when breakfast included some food which furnished a high quality protein.

EXPERIMENTAL

Fourteen college girls volunteered as subjects for this study for 36 days during the summer school session of 1948. They ranged in age from 17 to 23 years, in height from 5 ft. 2 in. to 5 ft. 10 in., in weight from 110 to 157 lb., and in surface area from 1.43 to 1.78 m^2. The girls lived together in a small dormitory and the research staff supervised the entire regimen. The activities of the different subjects were similar and constant. All were engaged in the sedentary or light work of an intensive school program and those who did not have afternoon laboratories usually had part-time office work.

The 36 days of the study were divided into two 18-day periods, designated as A and B. To facilitate rigid control of the amount and kinds of food and factors such as bulk and rate of digestion, a dietary plan using ordinary foods was made for a three-day period and this plan was repeated 12 times. This three-day diet was planned to meet the recommended allowances of the Food and Nutrition Board of the National Research Council ('48) for all nutrients except protein. Previous study had shown that the usual protein

intake of the subjects was between 60 and 63 gm daily. Because it was desirable not to change the state of protein nutrition of these subjects for a study of only 36 days, the diet was planned to furnish 62 gm of protein daily, 55 of which were from meat, milk, and milk products. During the first 9 days slight adjustments in calorie intake were made to insure that the subjects would remain at constant weight. The adjusted calorie intakes ranged from 1,950 to 2,200 and remained constant for each subject throughout the remaining 27 days of the study.

The difference between period A and period B was in the *distribution* of the animal protein, not in quantity or quality or food source. In period A the breakfast consisted of citrus fruit juice, bread made with water instead of milk, butter, jelly, and coffee or Postum. All of the animal protein was supplied in the noon and evening meals. The diet included 24 oz. of whole milk. During period A, 8 oz. of the milk were served at noon and 16 oz. were served at the evening meal. During period B, 8 oz. of the milk were served at each of the three meals, thus adding animal protein to the breakfast.

Despite rigid control of food selections and preparation the average daily nitrogen intake for period A was 9.78 gm as compared with 10.13 gm for period B; the difference of 0.35 gm was not statistically significant.

During the second 9 days of periods A and B a composite of all the food eaten by each subject at each meal was made, and all excreta for each subject were collected for analysis for nitrogen.

The urine collections for each subject for each 24-hour period were combined according to the times of voiding as follows: after breakfast, up to and including immediately before lunch; after lunch, up to and including immediately before dinner; after dinner, up to and including immediately before retiring at 11 P.M.; rising sample immediately before breakfast. Thus, 4 composites were analyzed for each 24-hour collection for each subject.

Carmine was given in the customary way to mark the stools. Stools for each 9-day period for each subject were combined for analysis.

Nitrogen was determined on acidified urine and on hydrochloric acid digests of food and feces for each subject by the Kjeldahl method.

RESULTS

The average daily nitrogen excretion for each subject for each period and the differences between the two periods are shown in table 1, together with statistical constants. The

TABLE 1

Average daily nitrogen excretion of each subject during two 9-day periods

SUBJECT	SURFACE AREA	PERIOD A [1]				PERIOD B [1]				DIFF. IN URINARY EXCRETION IN A AND B
		N excretion			N retention	N excretion			N retention	
		Urine	Feces	Total		Urine	Feces	Total		
	m^2	gm	gm	gm	gm	gm	gm	gm	gm	gm
11	1.43	8.58	0.75	9.33	0.45	8.15	0.50	8.65	1.48	0.43
3	1.49	8.16	1.03	9.19	0.59	8.01	0.72	8.73	1.40	0.15
15	1.49	8.86	0.72	9.58	0.20	8.50	0.78	9.28	0.85	0.36
Avg.	1.47	8.53	0.83	9.37	0.41	8.22	0.67	8.89	1.24	0.31
6	1.55	9.26	0.90	10.16	— 0.38	8.11	0.84	8.95	1.18	1.15
1	1.58	8.96	0.71	9.67	0.11	8.27	1.12	9.39	0.74	0.69
13	1.58	9.33	0.82	10.15	— 0.37	8.39	0.59	8.98	1.15	0.94
12	1.60	8.76	0.92	9.68	0.10	8.52	0.93	9.45	0.68	0.24
14	1.62	9.09	0.82	9.91	— 0.13	8.13	0.70	8.83	1.30	0.96
10	1.63	9.22	1.09	10.31	— 0.53	8.26	0.85	9.11	1.02	0.96
5	1.64	9.06	0.84	9.90	— 0.12	7.84	1.15	8.99	1.14	1.22
9	1.65	8.55	0.84	9.39	0.39	8.43	0.86	9.29	0.84	0.12
Avg.	1.61	9.03	0.87	9.90	— 0.11	8.24	0.88	9.12	1.01	0.78
2	1.74	9.54	0.45	9.99	— 0.21	8.74	0.77	9.51	0.62	0.80
8	1.77	9.17	0.64	9.81	— 0.03	9.01	0.77	9.78	0.35	0.16
4	1.78	9.80	0.52	10.32	— 0.54	8.27	1.15	9.42	0.71	1.53
Avg.	1.76	9.50	0.54	10.04	— 0.26	8.67	0.90	9.57	0.56	0.83
Average for all subjects	1.61	9.02	0.79	9.81	— 0.03	8.33	0.84	9.17	0.96	0.69
S.D.		0.410	0.171		0.189	0.290	0.190		0.329	
S.E.		0.1097	0.0457		0.050	0.0770	0.0504		0.0878	0.134

[1] The average daily nitrogen intake during periods A and B was 9.78 gm and 10.13 gm, respectively.

subjects have been arranged in ascending order of surface area. The total average daily urinary excretion of nitrogen for all subjects for period A was 9.02 gm with a S.D. of 0.41, and 8.33 gm with a S.D. of 0.29 for period B. The difference between the two periods was 0.69 with a S.E. of 0.134, which is highly significant according to the "t" test. Every subject excreted less urinary nitrogen during period B, when animal protein was present in every meal, than during period A when there was animal protein in only two meals. The difference ranged from 0.12 gm nitrogen daily for subject 9 to 1.53 gm for subject 4. The coefficients of variability for all subjects ranged from 5.23 to 16.52 for period A and from 4.24 to 10.14 for period B.

Analysis of the 4 urine composites which were collected for each subject in each 24 hours showed a significantly smaller excretion of nitrogen during the hours from after dinner until bedtime and from bedtime until breakfast time in period B as compared with period A. Analysis of variance for the two 9-day periods for the 14 subjects gave the following F values for the time of voiding stated: morning, 4.05 (significant at the 5% level); afternoon, 1.99; evening, 8.39 (significant at the 1% level); and night, 19.28 (significant at the 1% level).

The average daily fecal nitrogen for all subjects averaged 0.79 gm and 0.84 gm for periods A and B, respectively.

In period A, 8 of the 14 subjects had a total daily nitrogen excretion in excess of the intake; retentions ranged from —0.54 to 0.59 gm daily and averaged —0.03 gm for all subjects.

The situation was different during period B, when some animal protein was served in every meal. The daily nitrogen retention of the subjects ranged from 0.35 to 1.48 gm and averaged 0.96 gm.

The percentage of nitrogen absorbed and retained by each subject during periods A and B is shown in table 2. The average percentage absorption for all subjects was 91.9 for period A and 91.7 for period B. The average percentage re-

tention for all subjects was —0.4 for period A and 10.3 for period B.

The relation between the nitrogen metabolism figures and the surface area of the subjects is shown by averages given in tables 1 and 2 for groups of subjects with similar surface areas. There were three subjects with surface areas between 1.43 and 1.49 m^2; 8 with surface areas between 1.55 and 1.65 m^2; and three with surface areas between 1.74 and 1.78 m^2. There was no significant difference in the percentage

TABLE 2

Average daily percentage nitrogen absorption and retention of each subject during two 9-day periods

SUBJECT	SURFACE AREA	PERIOD A [1]		PERIOD B [1]		DIFF. IN URINARY EXCRETION IN A AND B
		Absorbed [2]	Retained [3]	Absorbed	Retained	
	m^2	%	%	%	%	%
11	1.43	92.3	5.0	95.1	15.4	10.4
3	1.49	89.5	6.7	92.9	14.9	8.2
15	1.49	92.6	2.2	92.3	9.1	6.9
Avg.	1.47	91.5	4.6	93.4	13.1	8.5
6	1.55	90.8	— 4.3	91.7	12.7	17.0
1	1.58	92.7	1.2	88.9	8.2	7.0
13	1.58	91.6	— 4.1	94.2	12.1	16.2
12	1.60	90.6	1.1	90.8	7.4	6.3
14	1.62	91.6	— 1.5	93.1	13.8	15.3
10	1.63	88.9	— 6.1	91.6	11.0	17.1
5	1.64	91.4	— 1.3	88.7	12.7	14.0
9	1.65	91.4	4.4	91.5	9.1	4.7
Avg.	1.61	91.1	— 1.3	91.3	10.9	12.2
2	1.74	95.4	— 2.3	92.4	6.6	8.9
8	1.77	93.5	— 0.3	92.4	3.7	4.0
4	1.78	94.7	— 5.8	88.7	7.9	13.7
Avg.	1.76	94.5	— 2.8	91.2	6.1	8.9
Avg. for all subjects	1.61	91.9	— 0.4	91.7	10.3	10.7

[1] The average daily nitrogen intake during periods A and B was 9.78 gm and 10.13 gm, respectively.

[2] Calculated according to the formula: $\frac{\text{gm N intake} - \text{gm N feces}}{\text{gm N intake}} \times 100$.

[3] Calculated according to the formula: $\frac{\text{gm N retained}}{\text{gm N absorbed}} \times 100$.

absorption of nitrogen in either period by the groups with different surface areas. In both periods the urinary nitrogen excretion increased, and the actual nitrogen retention and the percentage retention decreased in step-wise fashion with increasing surface area. For period A the correlation between the surface area and the percentage retention was significant at the 5% level, r equalling — 0.62, whereas for period B it was significant at the 1% level, r equalling — 0.71. There were no differences in the metabolism figures which could be related to age.

DISCUSSION

The differences in nitrogen metabolism during periods A and B are striking. Significance is added to the differences by the fact that in every case urinary excretion was less and percentage retention was greater in period B than in period A. The similarity in fecal excretion and percentage absorption of nitrogen in period A and period B suggests similar intestinal action during both periods.

If the lower urinary nitrogen excretion and higher retention in period B as compared with period A are considered to indicate better utilization, there was an advantage to these subjects in increasing the amount of protein in the breakfast and providing animal protein at every meal by transferring 8 oz. of milk from the dinner to the breakfast meal.

The present study was planned to simulate the fruit-coffee-roll breakfast and to find an effective yet simple and practical way to improve its contribution to the body's needs. It is for this reason that 8 oz. of milk were used as the addition to the breakfast. There are many factors not included in this pilot study which need to be considered in connection with the distribution of protein in the diet. In the present work the quantity as well as the quality of the protein of the breakfast was increased, although the daily protein intake remained constant. The results might have been different if the protein content of the breakfast had remained constant, or if it had been increased with protein of plant origin. With

lunch at noon and dinner at 6:00 o'clock, all the animal protein was eaten within a period of 6 to 7 hours, leaving a period of 17 to 18 hours when no animal protein was taken into the body. The nitrogen metabolism might have been different if animal protein had been given for breakfast and dinner instead of for lunch and dinner. Because of the time factor, lack of animal protein in the breakfast may impair nitrogen utilization more than its lack in the mid-day meal. Such questions as these are being investigated further.

The question arises as to whether dermal loss of nitrogen should be considered. The average of the mean daily air temperatures in period A was 75°F. and in period B 77°F., which suggests that loss of nitrogen in perspiration would have been similar during the two periods. Mitchell and Hamilton ('49) studied the dermal loss of 6 men under widely varying conditions and found no compensatory relationship between urinary and dermal loss of nitrogen.

It is not within the scope of this study to consider nitrogen requirements. The subjects were young adults who probably had need of some storage of nitrogen. This storage was accomplished during period B, when there was more and better quality protein in the breakfast than during period A. Neither can these findings be compared with other studies on nitrogen metabolism because it has not been customary to describe in the literature details of the amount and quality of protein in each of the daily meals served to research subjects.

In view of the nature and significance of the findings in the present study, it is possible that the distribution of quantity and kind of protein as well as the total daily amount of protein must be considered in determining protein requirements and utilization.

SUMMARY AND CONCLUSIONS

The nitrogen metabolism of 14 college-age women on a controlled diet which included no animal protein in the breakfast was compared with their nitrogen metabolism when the

breakfast included 8 oz. of milk, although the total protein intake remained unchanged.

During period A, when no animal protein was included in the breakfast, the average daily urinary excretion for all subjects was 9.02 gm nitrogen, S.D. 0.410, as compared with 8.33 gm, S.D. 0.290, during period B when animal protein from 8 oz. of milk was furnished with the breakfast. The difference in urinary excretion was 0.69, S.E. 0.134. In period A the average daily nitrogen absorption was —0.4% as compared with 10.3% in period B. The average daily nitrogen retention was —0.03 gm in period A as compared with 0.96 gm in period B.

In view of the nature and significance of the findings, it is possible that the distribution of quantity and kind of protein in the three meals, as well as the total daily amount of protein, must be considered in determining protein requirements and utilization.

ACKNOWLEDGMENT

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LITERATURE CITED

CANNON, P. R., C. H. STEFFEE, L. J. FRAZIER, D. A. ROWLEY AND R. C. STEPTO 1947 The influence of time of ingestion of essential amino acids upon utilization in tissue-synthesis. Fed. Proc., *6:* 390.

FOOD AND NUTRITION BOARD, NATIONAL RESEARCH COUNCIL, WASHINGTON 1948 Recommended Dietary Allowances, revised.

GEIGER, E. 1947 Experiments with delayed supplementation of incomplete amino acid mixtures. J. Nutrition, *34:* 97.

———— 1948 The role of the time factor in feeding supplementary proteins. Ibid., *36:* 813.

HARTE, R. A., J. J. TRAVERS AND P. SARICH 1948 Voluntary caloric intake of the growing rat. Ibid., *36:* 667.

HENRY, K. M., AND S. K. KON 1946 The supplementary relationships between the proteins of dairy products and those of bread and potato as affected by the method of feeding. J. Dairy Res., *14:* 330.

MITCHELL, H. H., AND T. S. HAMILTON 1949 The dermal excretion under controlled environmental conditions of nitrogen and minerals in human subjects, with particular reference to calcium and iron. J. Biol. Chem., *178:* 345.

SCHAEFFER, A. J., AND E. GEIGER 1947 Cataract development in animals with delayed supplementation of tryptophan. Proc. Soc. Exp. Biol. Med., *66:* 309.

THE IRON REQUIREMENT OF SIX ADOLESCENT GIRLS

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ONE FIGURE

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Information regarding the iron requirement of adolescent girls is meager. A dietary allowance of 15 mg per day has been recommended for girls 13 to 15 years of age by the National Research Council ('48). The recommendation is accompanied by a statement that "No data are available for the requirement during adolescence. The allowances recommended were estimated on the assumption that needs are greater than those of the adult."

The needs of children for iron differ from those of the adult because of growth. To choose a method which will accurately assess the requirements during growth is difficult. One possible method is to find the lowest iron intake which will support a good hemoglobin level. Since hemoglobin levels respond slowly to changes in the dietary intake, this method requires lengthy periods of study, preferably two or more years, for each level of iron intake. Johnston and Roberts ('42) applied this method to pre-adolescent children on one level of intake.

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Another method was used by Darby et al. ('47), who calculated the daily requirement of absorbable iron for children. The calculations were based on the percentage uptake of radioactive iron and on estimates of needs for storage and growth. This method for determining the dietary requirement depends upon the use of estimates of "absorbable" iron in foodstuffs. The author states: "at present there exists no reliable means of estimating the quantity of absorbable iron in a dietary; hence, the requirement of total dietary iron cannot now be ascertained by this method."

A method used by investigators in early years is the balance study. It was used on the assumption that a child would retain only the iron that he needed and that any excess would be excreted. The dietary level giving maximum retention was then taken to be the level of iron required. That assumption is now known to be erroneous. Once iron is absorbed by the body little is secreted back into the gastrointestinal tract, and the amount retained is not necessarily the amount needed. Thus the balance study in itself is not applicable to finding the iron requirement of children, but it is of value in determining the amount of iron which is retained from a given diet.

The method chosen for the present study had to be such that the needs of the adolescent girl who had reached the menarche would be taken into consideration. She needs to retain iron not only for growth but also for replacement of menstrual losses. If these needs are determined, the balance study can then be used to find the level of iron intake which will provide sufficient retentions to cover her needs.

Since there is no method available at present for determining the amount of iron which needs to be retained for growth, the decision was made to use a value based on estimates made by Heath and Patek ('37). They calculated the requirement of iron for growth on the basis of the annual gains in circulating and non-available tissue (extra-circulating) iron. The amount of iron which needs to be retained for replacement of menstrual losses can be determined directly by analysis of the menstruum.

PROCEDURE

The subjects were 6 girls, 13 and 14 years of age, all of whom had passed the menarche and were in excellent health; all had a history of good food habits. The age, height, weight and age of menarche of the subjects are given in table 1. They were housed and fed for 9½ weeks in the same building in which the laboratory is located.

Two levels of iron were administered in two consecutive 4-week periods preceded by an adjustment period of one week. During the adjustment period and the first 4-week period, 8 to 9 mg of iron were fed. This level was selected

TABLE 1

The age, height, weight and the age of menarche of the 6 subjects

SUBJECT	AGE [1]	HEIGHT	WEIGHT	AGE OF MENARCHE
	years	*in.*	*lb.*	*years*
A	14	64½	102½	13
B	14	62	125½	13
C	13	64½	111⅛	10
D	14	61⅜	109⅞	13
E	13	60⅜	79¼	12
F	13	67⅜	122¾	12

[1] Age to nearest birthday.

on the basis of previous work done on college women in this laboratory (Johnston, Frenchman and Boroughs, '48), in which an intake of 7 mg per day was found to be on the borderline of adequacy for the subjects. The slightly higher level was fed to the adolescent girls on the assumption that they would need more iron than the college woman. In case the first level was on the borderline or too low, a somewhat higher level of 11 to 12 mg of iron per day was chosen for the second 4-week period. One level of calcium, approximately 1 gm per day, was fed during the entire study to eliminate the possibility that a change in the level of calcium might affect the absorption of iron.

The basal diet was planned to be adequate in all known respects and to include a wide variety of natural foods. Two or more servings each of fruits and vegetables, one serving of meat and three glasses of milk for drinking (182 gm each) were included daily. To avoid a high phytic acid level in the diet and yet to include foods customarily eaten, a whole grain breakfast cereal, walnuts, shelled beans, cocoa and chocolate were used only once each week. Enriched bread was included daily in the basal diet.

To take care of differences in appetite and caloric needs, certain foods which contain little or no iron were allowed ad libitum. These foods were: unenriched bread; butter; grape jelly; sugar; lemonade made with distilled water; cookies made with unenriched flour, sugar and butter; and candy made from distilled water and other ingredients containing negligible amounts of iron. A limited number of bottles of a low-iron cola beverage were allowed each week. The weights of all foods eaten ad libitum were recorded. While these foods were low in iron, the quantity eaten by the subjects made the total iron intake vary somewhat from week to week and from subject to subject.

To plan the diet for the first experimental period during which the food contained 8 to 9 mg of iron per day offered no problem and required only the omission of foods containing extremely high quantities of iron, such as liver. One serving each of spinach, green peas and green beans was included each week. The level of iron was increased to between 11 and 12 mg per day by increasing the size of servings of some foods high in iron such as meat, eggs and vegetables, and by the addition to the original diet of a few foods rich in iron. These foods were dried apricots, dates and a small serving of liver sausage.

The menu was planned on a weekly basis and repeated in successive weeks. The basal diet was carefully controlled so that the level of iron in it would be the same for all 4 weeks. Frozen fruits and vegetables and most of the fresh vegetables were obtained in sufficient quantity at the beginning of the

study to carry through the 9 weeks. Where possible, other items were purchased in one lot for the entire study. All fat was trimmed from the cooked meats and the lean portion only weighed. The foods were prepared and served with particular attention paid to avoiding the possibility of contamination; distilled water was used for cooking and drinking; all cooking utensils were either of aluminum or glass and the kitchen tools were of stainless steel.

Composites of foods in the basal diet were made each week. Aliquot portions equal to one-fifth of the serving weight were composited independently by two people. These were mixed in a mechanical blender, treated with concentrated HCl, heated and bottled. The milk, bread, biscuits, rolls, cola drinks, lemonade, grape jelly and cookies were analyzed separately. The feces were marked by using carmine capsules. Weekly composites of feces were mixed in a blender, acidified with HCl, heated and stored in glass. Urinary collections were made for a weekly period on each level of intake and an aliquot portion was acidified with HCl and stored. Menses were collected during and after the experimental period for a minimum of 4 menstrual periods for each subject. Tampons obtained in one lot were used for collection of the menses. The used tampons were allowed to stand several hours in redistilled water, acidified with HCl, heated to form a slurry and stored in glass. A correction was made for the iron in the tampon. A record was kept of the cycle lengths for each subject.

The foods, feces, urine and menses were each wet-ashed with nitric and sulfuric acid and the nitrosyl-sulfuric acid was hydrolyzed by boiling with water according to the method of Roberts, Beardsley and Taylor ('40). Iron was determined by the thiocyanate method of Stugart ('31), and the intensity of the color read in an Evelyn photoelectric colorimeter.

Venous blood samples were taken three times during the study to determine hemoglobin values, red blood cell counts and serum iron levels. They were taken at 4 P.M., after a period of inactivity to eliminate the possible effects of exercise

on the level of these components of blood. Each determination was made in duplicate. For serum iron 2 ml samples of serum were analyzed; the protein was precipitated by the method of Kitzes, Elvehjem and Schuette ('44) and the iron was determined by the *o*-phenanthroline method of Saywell and Cunningham ('37). Hemoglobin was determined by the method of Evelyn ('36). Cell counts were made in duplicate on blood from each of two pipettes.

RESULTS AND DISCUSSION

Daily intake, retention and absorption

The average daily intake of iron for the first 4-week period for all the subjects was 8.6 mg, and ranged from 8.0 to 9.6 mg (table 2). On the diet with the higher level of iron, the average daily intake was 11.7 mg and ranged from 10.9 to 13.3 mg (table 2). Subject F, the tallest girl, ate the largest amount of the food allowed ad libitum and therefore received the most iron; subject E, the shortest girl, received the least iron because she ate little in addition to the basal diet.

Retentions of iron by the subjects represent the difference between that in the food intake and that in the feces and urine (table 2). On an average intake of 8.6 mg per day, average iron retentions for each subject were 0.80, 0.86, 1.22, 1.23, 1.42 and 1.54 mg per day. When the average intake was 11.7 mg per day, the average retentions for each subject were 0.33, 1.15, 1.54, 1.76, 2.10 and 2.22 mg per day. On the diet containing the larger amount of iron, higher retentions occurred for all except subject B, whose retention was so much lower than that of the other subjects and than her own first period that it appeared to be atypical. No explanation could be found for the sudden drop in her retention from 1.54 mg per day during the first period to 0.33 mg in the second period.

On a percentage basis, the subjects retained 9, 9, 14, 14, 18 and 19% of the iron from the diet with 8.6 mg per day, and 3, 10, 13, 16, 16 and 19% from the diet with 11.7 mg per day. The average percentage retentions for all the subjects were

TABLE 2

The excretion and retention of iron by 6 subjects on two levels of iron intake

SUBJECT	ADJUSTMENT PERIOD	FIRST PERIOD					SECOND PERIOD				
	Week I	Week II	Week III	Week IV	Week V	Average	Week VI	Week VII	Week VIII	Week IX	Average
A:											
Fe in food (mg/day)	8.20	8.06	8.77	8.73	8.36	8.48	11.28	11.78	11.74	11.15	11.49
Fe in feces and urine (mg/day)		7.41	7.47	7.80	8.03	7.68	9.50	10.66	10.67	10.51	10.34
Fe retained (mg/day)						0.80					1.15
B:											
Fe in food (mg/day)	7.98	8.05	8.38	8.14	7.76	8.08	10.77	10.94	11.09	10.62	10.86
Fe in feces and urine (mg/day)		7.42	5.48	5.60	7.66	6.54	10.11	10.78	10.40	10.82	10.53
Fe retained (mg/day)						1.54					0.33
C:											
Fe in food (mg/day)	8.18	8.24	8.77	8.81	8.26	8.52	11.60	11.81	12.41	11.50	11.83
Fe in feces and urine (mg/day)		6.89	7.46	7.14	7.69	7.29	10.29	9.65	10.81	10.40	10.29
Fe retained (mg/day)						1.23					1.54
D:											
Fe in food (mg/day)	8.21	8.27	8.89	9.41	9.29	8.96	11.91	11.92	12.25	11.78	11.96
Fe in feces and urine (mg/day)		6.82	6.60	7.45	10.11	7.74	10.01	9.01	9.93	10.03	9.74
Fe retained (mg/day)						1.22					2.22
E:											
Fe in food (mg/day)	7.89	7.94	8.39	8.08	7.66	8.02	10.82	11.14	11.08	10.62	10.92
Fe in feces and urine (mg/day)		6.15	7.05	7.12	6.08	6.60	9.02	8.87	9.67	9.08	9.16
Fe retained (mg/day)						1.42					1.76
F:											
Fe in food (mg/day)	8.32	8.57	9.51	10.23	10.10	9.60	13.61	13.51	13.59	12.36	13.26
Fe in feces and urine (mg/day)		8.77	8.25	8.90	9.05	8.74	11.18	10.86	12.63	9.99	11.16
Fe retained (mg/day)						0.86					2.10

14 and 13%, respectively, of the iron from the two diets. The unusually low retention of subject B from the higher level of intake lowered the average retention for the second period. The percentage of absorption also was calculated. For this, the iron in the feces was subtracted from that in the food intake. Because the amount of iron in urine is so small, the percentage absorption values were the same as the retention values. These values are similar to those in the literature for girls younger than these and for women. Darby et al. ('47) reported that girls 10 years of age absorbed 14.5% of a test dose of radioactive iron. College women studied by Johnston, Frenchman and Boroughs ('48) absorbed 11% from a normal mixed diet containing 7 mg of iron per day, while adult women studied by Widdowson and McCance ('42) absorbed 12.5% from a diet in which white bread made up 40 to 50% of the calories and the remainder were provided by a mixed diet.

Blood picture

The blood picture of the subjects as indicated by hemoglobin values, red blood cell counts and serum iron levels was satisfactory. Hemoglobin values were 13.2 gm per 100 ml of blood or above, and red blood cell counts were all 4.35 $\overline{M}$ per mm^3 or above (table 3). These values are above the average reported by Leichsenring, Donelson and Wall ('41) for girls of this age. Serum iron levels were variable, ranging from 57 to 97 μg per 100 ml of serum (table 3). These levels are similar to those observed in the serum of other girls of this age in work done in this laboratory (unpublished). The highly satisfactory blood picture suggests that the 6 subjects were absorbing iron in a normal fashion, since all probably had normal stores of iron.

Menstrual losses

Average menstrual losses for 4 or more periods varied widely among the subjects and ranged from 6.12 to 50.11 mg per period (table 4). Considerable variation in iron losses

per period for each girl was observed (fig. 1). Losses for subject A, for example, ranged from 1.73 to 13.83 mg per period. In 4 of the 6 subjects, the first menses collected during the study had the least iron (fig. 1). Whether or not the unusual situation contingent upon being a part of the experiment somehow affected the menstrual loss during the first collection period is a question. In a search of the literature no studies were found regarding iron losses in the menses of

TABLE 3

Hemoglobin values, red blood cell counts and serum iron levels for the 6 subjects determined during the last week of each period

SUBJECT	A	B	C	D	E	F
			Hemoglobin in gm per 100 ml of blood			
Adjustment period	13.7	13.4	13.8	13.0	15.2	14.3
8.6 mg-intake period	14.2	13.8	13.6	13.3	14.8	13.9
11.7 mg-intake period	13.3	13.4	13.6	13.2	14.6	14.0
Average	13.7	13.5	13.7	13.2	14.9	14.1
			Red blood cell counts in $\overline{M}$ per mm³			
Adjustment period	4.87	4.35	4.50	4.24	4.88	4.65
8.6 mg-intake period	4.90	4.38	4.43	4.38	4.80	4.38
11.7 mg-intake period	4.66	4.61	4.68	4.42	4.81	4.44
Average	4.81	4.45	4.54	4.35	4.83	4.49
			Iron in μg per 100 ml of serum			
Adjustment period	105	86	76	58	74	72
8.6 mg-intake period	92	76	75	64	76	70
11.7 mg-intake period	94	79	44	50	85	72
Average	97	80	65	57	78	71

girls 13 and 14 years of age, but iron losses during menstruation have been studied in adult women. Frenchman and Johnston ('49) reviewed the literature on this subject and found that reported iron losses in the menses of 184 women ranged from 2.28 to 78.96 mg per period. The range of iron losses for the 6 girls in this study was from 0.59 to 74.38 mg per period, which was as large as that for the adult women studied by others.

In order to calculate the amount of iron needed daily to replace menstrual losses, information regarding the length of the menstrual cycle is necessary. Considerable variation was observed in the length of the cycles of the present subjects. The average length of the cycle of the 6 girls was 29.8 days and ranged from 21 to 41 days (table 4). The average number of days in these cycles is somewhat less than that reported by Engle and Shelesnyak ('34), who found an average of 33.9 days in the menstrual cycles of 100 young girls.

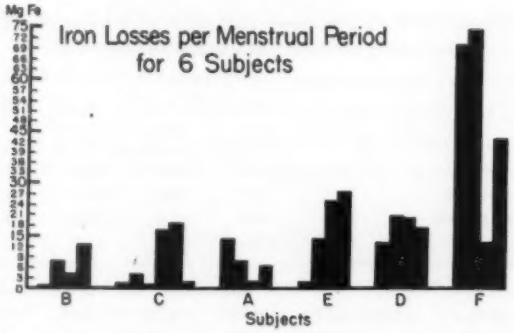

Fig. 1 Each bar represents the milligrams of iron in the menstruum from one menstrual period.

TABLE 4

Daily retention of iron required by the 6 subjects for replacement of menstrual losses

SUBJECT	NO. OF MENSTRUAL PERIODS	LENGTH OF CYCLE [1]		AVERAGE FE LOSS PER PERIOD	DAILY RETENTION OF FE NEEDED FOR REPLACEMENT [2]
		Average	Range		
		days	*days*	*mg*	*mg*
A	4	41.0	35–46	7.46	0.18
B	4	28.0	24–31	6.12	0.22
C	6	21.0	13–31	7.28	0.35
D	4	31.5	29–33	17.78	0.56
E	4	23.3	20–25	16.94	0.73
F	4	33.7	19–43	50.11	1.49
Average		29.8		17.62	0.59

[1] The length of the cycle is the number of days from the beginning of one menstrual period to the beginning of the next.

[2] This value was computed as follows: $\frac{\text{Average iron loss per period in mg}}{\text{Average length of the cycle in days}}$.

According to Reymert and Jost ('47), who studied 100 girls 10 to 18.5 years of age, young girls have more irregular menstrual cycles than do older ones; this irregularity, according to Engle and Shelesnyak ('34), decreases steadily with increasing elapsed time since the menarche. Among the girls in this study, subject F, who had passed the menarche shortly before the beginning of the study, had the most irregular cycles; they ranged from 19 to 43 days in length. In view of the variability observed in the menstrual picture, the menstruum from 4 periods is probably the minimum which should be collected and analyzed from girls of this age to obtain a representative average value. Analysis of the menstruum from more than 4 periods would be highly desirable.

To find the daily retention of iron needed for replacement of menstrual losses, the following formula was applied: $\frac{\text{average iron loss per period in mg}}{\text{average length of cycle in days}}$. Using this formula, the daily retentions needed by the 6 subjects to cover menstrual losses were 0.18, 0.22, 0.35, 0.56, 0.73 and 1.49 mg (table 4). For replacement of menstrual losses, subject F needed to retain 8 times the amount of iron needed by subject A.

Growth

To find the retention required for growth, the estimated values of Heath and Patek ('37) for the yearly retention of iron needed by girls were used. For 5 subjects the value 145 mg, for girls between the ages of 13 to 14, was used; for subject A the value for the next age group, 170 mg, was used because she had passed her 14th birthday. The values were divided by 365 to convert them into the daily retentions required; these were 0.40 and 0.46 mg per day. Obviously these are average values and girls who are growing faster or slower than the average need more or less. According to Heath and Patek ('37) these values were based on estimates of annual gains of circulating iron and the non-available tissue iron (extra-circulating iron) which increases with growth. The annual gains of circulating iron were estimated on the basis

of the gain in total circulating hemoglobin. This in turn was based on the average hemoglobin in total blood volume for different ages. The annual gain in extra-circulating or unavailable tissue iron was calculated from the arbitrary standard of 5 mg of iron per kilogram of body weight. The authors state that their data are only approximate but that they supply specific information which will be helpful in reaching logical conclusions.

Adequacy of the dietary intake

The adequacy of the two levels of dietary intake can be assessed by comparing the retentions observed with the retentions needed by the subjects. By adding together the amounts needed for replacement of menstrual losses and the

TABLE 5

Estimated retentions required and observed retentions on two levels of iron intake

SUBJECT	ESTIMATED RETENTION REQUIRED [1]	OBSERVED RETENTIONS	
		Average intake 8.6 mg	Average intake 11.7 mg
	mg/day	*mg/day*	*mg/day*
A	0.64	0.80	1.15
B	0.62	1.54	0.33
C	0.75	1.23	1.54
D	0.96	1.22	2.22
E	1.13	1.42	1.76
F	1.89	0.86	2.10
Average	1.00	1.18	1.52

[1] The estimated retention required is the amount of iron needed for growth and the amount needed for replacement of menstrual losses.

amounts estimated as being required for growth, the following are the minimum retentions required by the 6 subjects: 0.62, 0.64, 0.75, 0.96, 1.13 and 1.89 mg per day (table 5). Wide differences in the retentions required are apparent. They are due mainly to differences in menstrual losses. Three subjects had menstrual losses which were very small, so small that the amount of iron needed to replace these losses was less than the amount estimated as needed for growth.

When the values for the 6 subjects are averaged, the daily retention required is 1.00 mg and the observed daily retentions from the two diets are 1.18 and 1.52 mg (table 5). Thus the average retentions observed on the two diets were greater than those required. From these averages one might conclude that the intake of 8.6 mg of iron per day was adequate for these girls. The average should not be used alone, however, in drawing a conclusion. Since only 6 cases make up these averages, the performance of the individuals should be examined more closely. The average intake of the first period, 8.6 mg per day, was not sufficient to cover the needs of subject F. Her menstrual losses were larger than those of the others, hence her needs were larger. While her losses were large, comparison of them with those of women reported in the literature leads to the belief that such losses are not uncommon. For the other 5 subjects, this dietary intake was possibly sufficient if the criterion for adequacy is merely that the retentions observed are larger than the individual estimated required retentions (table 5). The size of the margin between the observed retentions and those required, however, should also be examined. This margin should be large enough to take care of any small additional needs such as those for replacement of iron lost in sloughed-off skin cells and hair, or those which might be due to an accelerated growth rate. The margin observed for three subjects on this dietary intake was small.

The diet containing an average of 11.7 mg of iron daily was adequate for these girls with the exception of subject B, whose retention appeared to be atypical. For 4 subjects, a wide margin of safety was observed which should easily take care of any small additional needs for iron. During this period subject F, who exhibited the largest menstrual losses, retained enough to cover her total needs and to have a margin of safety. Her actual intake was 13.3 mg per day, which was above the average for the group.

Any recommendation for a dietary intake of iron based on only 6 cases should be a generous one. The possibility should be recognized that the retentions found on the intakes used

in this study may have been larger than might occur in actual practice when the diet was not as adequate in other nutrients as this one. Because one subject required approximately 13 mg, a desirable recommendation would be 12 to 13 mg per day. Many girls, of course, would not need an intake of iron that high and an occasional girl might need more. Possibly after more subjects of this age have been studied on similar and on different diets, a lower intake can safely be recommended. The allowance of 15 mg per day recommended by the National Research Council ('48), which had to be made without experimental evidence as a basis, is probably higher than necessary.

SUMMARY

Six girls, 13 and 14 years of age, all of whom had passed the menarche, were fed a controlled diet for 9 weeks. Two levels of iron were fed in two consecutive 4-week periods preceded by an adjustment period of one week. During the first 4-week period the average intake was 8.6 mg of iron per day, and during the second period the intake was raised to 11.7 mg daily.

The iron required by the 6 subjects was estimated by adding together the amount needed to replace menstrual losses as determined by analysis and the amount needed for growth based on the estimates of Heath and Patek ('37). The minimum required retentions were 0.62, 0.64, 0.75, 0.96, 1.13 and 1.89 mg per day, or an average of 1.00 mg per day. On an intake of approximately 8.6 mg per day, retentions for the 6 girls were 0.80, 0.86, 1.22, 1.23, 1.42 and 1.54 mg per day, or an average of 1.18 mg daily. Five of the 6 retentions on this intake were larger than the probable minimum required retentions; the retention of one subject was inadequate. On an intake of approximately 11.7 mg per day, retentions for the 6 girls were 0.33, 1.15, 1.54, 1.76, 2.10 and 2.22 mg per day, or an average of 1.52 mg daily. On the higher dietary intake the retentions of the subjects were larger than the minimum required retentions, with a wide margin of safety, except for one subject, whose retention appeared to be atypical

On the basis of the 6 cases in this study, a dietary intake of 12 to 13 mg per day is recommended for girls of this age until more cases have been studied. The allowance of 15 mg per day recommended by the National Research Council ('48), which had to be made without experimental evidence as a basis, is probably higher than necessary.

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LITERATURE CITED

DARBY, W. J., P. F. HAHN, M. M. KASER, R. C. STEINKAMP, P. M. DENSEN AND M. B. COOK 1947 The absorption of radioactive iron by children 7–10 years of age. J. Nutrition, *33:* 107.

ENGLE, E. T., AND M. C. SHELESNYAK 1934 First menstruation and subsequent menstrual cycles of pubertal girls. Human Biol., *6:* 431.

EVELYN, K. A. 1936 A stabilized photoelectric colorimeter with light filters. J. Biol. Chem., *115:* 63.

FOOD AND NUTRITION BOARD, NATIONAL RESEARCH COUNCIL 1948 Recommended Dietary Allowances. National Research Council Reprint and Circular Series No. 129.

FRENCHMAN, R., AND F. A. JOHNSTON 1949 The relation of menstrual losses to the iron requirement. J. Am. Diet. Assoc., *25:* 217.

HEATH, C. W., AND A. J. PATEK, JR. 1937 The anemia of iron deficiency. Medicine, *16:* 267.

JOHNSTON, F. A., R. FRENCHMAN AND E. D. BOROUGHS 1948 The absorption of iron from beef by women. J. Nutrition, *35:* 453.

JOHNSTON, F. A., AND L. J. ROBERTS 1942 The iron requirement of children of the early school age. Ibid., *23:* 181.

KITZES, G., C. A. ELVEHJEM AND H. A. SCHUETTE 1944 The determination of blood plasma iron. J. Biol. Chem., *155:* 653.

LEICHSENRING, J. M., E. G. DONELSON AND L. M. WALL 1941 Studies of blood of high school girls. Am. J. Dis. Child., *62:* 262.

REYMERT, M. L., AND H. JOST 1947 Further data concerning the normal variability of the menstrual cycle during adolescence and factors associated with age of menarche. Child Develop., *18:* 169.

ROBERTS, H. L., C. L. BEARDSLEY AND L. V. TAYLOR, JR. 1940 Determination of iron in liquid food products. Ind. Eng. Chem., Anal. Ed., *12:* 365.

SAYWELL, L. G., AND B. B. CUNNINGHAM 1937 Determination of iron. Ibid., *9:* 67.

STUGART, R. 1931 Determination of iron in milk and other biological materials. Ibid., *3:* 390.

WIDDOWSON, E. M., AND R. A. MCCANCE 1942 Iron exchanges of adults on white and brown bread diets. Lancet, *1:* 588.

THE GROWTH-PROMOTING EFFECT ON THE RAT OF SUMMER BUTTER AND OTHER FATS

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While the present authors were carrying out experiments on rats fed a fat-free diet for the purpose of establishing the existence or non-existence of fat soluble factors other than those already known, the work of Boer et al. ('47a, b) on vaccenic acid as a growth-promoting factor was published. As we at that time had been unable to detect the presence of any heretofore unrecognized fat soluble factors essential for growth in the rat, we decided to determine to what extent the findings of Boer et al. would influence our further work.

The presence in butter of unidentified nutritional factors not present in vegetable fats has received the attention of many investigators in recent years. Boutwell et al. ('41, '43) concluded that the superior growth-promoting properties of butterfat as compared to certain vegetable oils is probably due to a saturated compound present in butterfat in small amounts. In a subsequent paper these authors found that the superior growth of rats fed butterfat as compared to that of rats fed corn oil was manifest only when the sole carbohydrate in the diet was lactose. Brown and Bloor ('45) investigated the nutritive value of fractions of fatty acids of butter and whole butterfat and found that the efficiency of a whole butterfat diet in producing gains in body weight of rats was either matched or improved upon by the liquid fatty acid fractions. Henderson et al. ('45) and Jack et al. ('45), in their nutritional studies on milk fat, found that a

diet containing milk fat had no advantage in so far as rat growth is concerned over one containing triolein prepared from olive oil.

Boer ('41) concluded that butterfat contains a new factor necessary for normal growth in rats. Later Boer and Jansen ('42) confirmed the presence in butter of a substance needed for normal growth in rats which is absent from, or present in smaller amounts in, margarine, peanut oil, olive oil, and other fats. The same group (Boer et al., '46) also reported that summer butter, which they define as butter from cows on green pasture, contains a fat soluble substance different from all known vitamins which promotes growth in rats, and that this factor is found in the fatty acid fraction with more than 14 carbon atoms. In more recent work (Boer et al., '47a, b) this factor was finally identified as vaccenic acid.

Deuel et al. ('44) reported that no difference in the growth of weanling rats was noted at any time over a 12-week period when they were fed a butter diet in contrast with results obtained with diets containing corn, cottonseed, peanut or soybean oils, or margarine. In further work, Deuel et al. ('48)[1] found that no difference in the rate of growth of male and female rats was noted over a 6-week period when the diet contained butterfat or cottonseed oil and, further, that no stimulating effect on growth was produced by the administration of vaccenic acid or partially hydrogenated china-wood oil to rats on a rapeseed oil diet. They concluded that vaccenic acid plays no specific role in the growth of the rat.

Recently, von Euler and von Euler ('48)[2] have confirmed the findings of Deuel et al. by observing the growth of young rats through a 25-day test period. In earlier work von Euler et al. ('43) found no evidence of the presence of any special nutritive factors in butterfat which were not also present in margarine. Nath et al. ('48a)[3] separated September butterfat into a liquid and a solid fraction by low temperature sol-

[1] Published since completion of the present experiments.

[2] See footnote 1.

[3] See footnote 1.

vent fractionation. The liquid fraction gave a growth rate in weanling rats superior to that produced by whole butterfat or corn oil, while the solid fraction supported a significantly slower growth rate. In later work ('48b)[4] this group studied the alleged growth-promoting property of vaccenic acid and concluded that this fatty acid does not have any growth-promoting effects on rats fed diets containing corn oil or olive oil.

To clarify the apparent conflict which exists between the results of Deuel et al. and other authors on the one hand, and those of Boer et al. on the other, as well as to get a further insight into the requirements of the rat for as yet unidentified fat soluble growth factors, the following experiments were undertaken.

EXPERIMENTAL

The rats used in these experiments were male albinos of the Sprague-Dawley strain. At weaning they were divided into groups of 10 each balanced as to weight, placed in individual wire-floored cages and given the diets shown in table 1 and distilled water ad libitum. The fat soluble vitamin supplements were administered three times weekly in small glass supplementing dishes. Individual weights of the rats were obtained at the start of the experiment and once weekly for a feeding period of 70 days. The feeds and the vitamin supplements were stored until used in a refrigerator under nitrogen. At the end of the experiment the rats were killed, eviscerated, their body length determined (from nose to base of tail), and ground in a meat grinder. Composite samples from each group were analyzed for fat by a cold ether extraction of the finely comminuted carcasses in the presence of an excess of anhydrous sodium sulfate.

The ethyl linoleate used in these experiments was prepared from cottonseed oil by the bromination method of McCutcheon ('42) and stored under a nitrogen atmosphere in a refrigerator until used. The casein was prepared by an exhaustive

[4] See footnote 1, page 84.

TABLE 1

Experimental diets fed to different groups of rats

INGREDIENT	GROUP				
	1	2	3	4	5
	% in diet				
Fat free basal diet [1]	80.0	80.0	80.0	80.0	90.0
Vitamin B complex supplement [2]	10.0	10.0	10.0	10.0	10.0
Summer butterfat	10.0	...	...	...	...
Oleomargarine fat (Nucoa)	...	10.0	...	...	...
Olive oil	...	...	10.0	..	...
Cottonseed oil	...	...	...	10.0	...
Composition of fat soluble supplement	*Daily dose per rat*				
Ethyl linoleate	0.2 ml	0.2 ml	0.2 ml	..	0.2 ml
Fat soluble vitamins	[3]	[3]	[3]	[3]	[3]

[1] The fat free basal diet had the following composition: Ether extracted vitamin test casein, General Biochemicals, Inc., 30.0%; Sure's salts no. 1 ('41) 4.5%; cystine, C.P., 0.3%; sucrose, 65.2%; 2-methyl, 1-4 naphthoquinone, 1 μg per gram of diet.

[2] The vitamin B complex supplement had the following composition: Skelly-solve F extracted rice bran concentrate, National Oil Products Co., 966 gm; riboflavin, 0.10 gm; calcium pantothenate, 0.25 gm; inositol, 3.50 gm; choline chloride, 5.00 gm; distilled water to make 1,000 ml.

[3] For groups 1, 2, 3, and 5 the following daily amounts of fat soluble vitamins were fed, dissolved in the ethyl linoleate: crystalline vitamin D_3, 25 I.U.; crystalline beta carotene, 50 μg; alpha-tocopherol, 1.0 mg. In the case of group 4, the same amounts of the vitamins were fed, dissolved in a daily dose of 0.2 ml of cottonseed oil.

ether extraction of vitamin test casein [5] which, when received, had already been extracted repeatedly with ethyl alcohol during its manufacture. The vitamin B complex supplement was prepared from rice bran concentrate [6] by diluting it with distilled water in the ratio of one to three and liquid-extracting this mixture with petroleum ether (Skelly-solve F) until a fat free extract was obtained as revealed by the turbidity test of Bloor ('14).

[5] Obtained from General Biochemicals, Inc.

[6] Obtained from National Oil Products Co., Harrison, N. J.

DISCUSSION AND RESULTS

One of the main difficulties encountered in obtaining reliable results is the need to produce a fat free basal diet, particularly when growth-promoting substances that may be active in minute amounts are involved. Mackenzie et al. ('39) showed the difficulties involved in obtaining fat free diets by solvent extraction of yeast and casein. Commercial sucrose was shown by these workers to be a fat free source of carbohydrate. In order to eliminate the possibility of fat contamination by yeast (commonly used as a B complex supplement in low fat diets) and the carbohydrate-containing fraction of the diet, we decided to use fat-extracted rice bran concentrate as the source of vitamin B complex factors and commercial sucrose as the main carbohydrate.

The basal diet, when tested on a group of rats in earlier experiments, produced the typical essential fatty acid deficiency symptoms described by Martin ('39) within 90 days. Since the casein in our basal diet is the only ingredient which, according to our views, may still contain small amounts of fat, it would seem reasonable to assume that an essentially fat free diet could be compounded by replacing casein with a protein hydrolysate which can be made absolutely fat free. This would of course pose the additional problem of producing a hydrolysate that supports normal growth. This problem is now being further investigated in this laboratory.

The results given in table 2 show that there are no heretofore unrecognized fat soluble growth-promoting factors for the rat (vaccenic acid or others) present in summer butterfat which are not also present in margarine fat [7] and cottonseed oil to the same extent as measured by growth response. This conclusion is supported by comparison of weight gains as well as mean body lengths of the rats. That the formation of adipose tissue in the carcasses of the rats does not influence these conclusions is seen by a comparison of the carcass fat content determined for each group. Group 4, which was

[7] Nucoa.

fed cottonseed oil, showed the largest gain both as to weight and body length, but a statistical evaluation [8] of the data proved the difference to be non-significant. Cottonseed oil, however, gave a significantly greater growth response than did olive oil. Group 5, which received no other fat than ethyl linoleate, showed a significantly lower response both as to growth and body length than the groups that were fed

TABLE 2

Results of feeding experiment

CATEGORY OF INTEREST	GROUP				
	1	2	3	4	5
Mean daily weight gain per rat in grams from 30 through 100 days of age	4.28	4.27	3.99	4.42	3.63
Standard error of the mean	0.141	0.104	0.0950	0.124	0.0890
Average daily food intake in grams per rat	17.2	17.3	16.4	19.7	20.2
Mean body length in millimeters at 100 days	243.0	245.0	241.4	246.7	235.9
Standard error of the mean	1.36	1.14	1.31	1.78	1.74
Per cent fat in eviscerated carcasses	14.91	14.56	15.89	14.26	12.47

summer butterfat, margarine fat, and cottonseed oil. This would seem to indicate that all the fats fed in these experiments stimulated the growth of rats when added to a fat free diet. These results are in accord with those of Deuel et al. ('47).

Our conclusions with reference to vaccenic acid are in contradiction to the results of Boer et al. ('47a, b), who claim

[8] Our criterion for significant difference was as follows: $\frac{m_1 - m_2}{\sqrt{E_1^2 + E_2^2}}$, where m_1 and m_2 are the two mean results and E_1 and E_2 are their respective standard errors. If the figure derived from this expression was two or more, the difference was considered significant.

that summer butterfat contains a rat growth-promoting substance, later identified by them as vaccenic acid, present in smaller amounts in, or absent from, certain vegetable oils. In their work on the vaccenic acid content of various fats and oils Geyer et al. ('47) have shown cottonseed oil to contain no vaccenic acid. Therefore, since in our work the group of rats fed cottonseed oil showed the greatest gain in weight and body length, we may conclude that vaccenic acid is of no significance as a growth-promoting factor. These conclusions are in accord with those of Deuel et al. ('48), von Euler and von Euler ('48), and Nath et al. ('48b).

SUMMARY

Rat growth experiments were conducted over a 70-day period in which summer butterfat, margarine fat, cottonseed oil, and olive oil were added at levels of 10% to an otherwise fat free basal ration capable by itself of producing essential fatty acid deficiency symptoms. Using growth and body length measurements as criteria, it is concluded that there are no heretofore unrecognized fat soluble growth factors such as vaccenic acid present in summer butterfat which are not also present to the same extent in margarine fat[9] and cottonseed oil. The addition of each of the above fats to a fat free diet produced a significant growth stimulation.

ACKNOWLEDGMENTS

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[9] See footnote 7, page 87.

LITERATURE CITED

BLOOR, W. R. 1914 A method for the determination of fat in milk. J. Am. Chem. Soc., *36:* 1300.

BOER, J. 1941 A new growth factor in butterfat. Acta brevia neerland., *11:* 180. Through Chem. Abstr., *36:* 126 (1942).

BOER, J., AND B. C. P. JANSEN 1942 Studies on the nutritional value of butter in comparison with other fats supplemented with various amounts of vitamin A and D. Arch. néerland. physiol., *26:* 1. Through Chem. Abstr., *37:* 4444 (1943).

BOER, J., B. C. P. JANSEN AND A. KENTIE 1946 Further investigations on a fat-soluble growth-promoting substance present in summer butter. Nature, *158:* 201.

———— 1947a On the growth-promting factor for rats present in summer butter. J. Nutrition, *33:* 339.

BOER, J., B. C. P. JANSEN, A. KENTIE AND H. W. KNOL 1947b The growth promoting action of vaccenic acid. Ibid., *33:* 359.

BOUTWELL, R. K., R. P. GEYER, C. A. ELVEHJEM AND E. B. HART 1941 The effect of hydrogenation on the nutritive value of the fatty acid fractions of butter fat and of certain vegetable oils. J. Dairy Sci., *24:* 1027.

———— 1943 Further studies on the growth promoting value of butterfat. Ibid., *26:* 429.

BROWN, E. F., AND W. R. BLOOR 1945 The nutritive value of the fatty acids of butter including their effect on the utilization of carotene. J. Nutrition, *29:* 349.

DEUEL, H. J., JR., S. M. GREENBERG, E. E. STRAUB, D. JUE, C. M. GOODING AND C. F. BROWN 1948 Studies on the comparative nutritive value of fats. X. On the reputed growth promoting activity of vaccenic acid. Ibid., *35:* 301.

DEUEL, H. J., JR., E. R. MESERVE, E. STRAUB, C. HENDRICK AND B. T. SCHEER 1947 The effect of fat level of the diet on general nutrition. I. Growth, reproduction and physical capacity of rats receiving diets containing various levels of cottonseed oil or margarine fat ad libitum. Ibid., *33:* 569.

DEUEL, H. J., JR., E. MOVITT, L. F. HALLMAN AND F. MATTSON 1944 Studies of the comparative nutritive value of fats. I. Growth rate and efficiency of conversion of various diets to tissue. Ibid., *27:* 107.

GEYER, R. P., H. B. NATH, V. H. BARKI, C. A. ELVEHJEM AND E. B. HART 1947 The vaccenic acid content of various fats and oils. J. Biol. Chem., *169:* 227.

HENDERSON, J. L., E. L. JACK, S. LEPKOVSKY AND D. F. REID 1945 Nutritional studies of milk fat. I. The growth of young rats fed milk fat and certain synthetic glycerides as supplements to a fat-free diet. J. Nutrition, *30:* 169.

JACK, E. L., J. L. HENDERSON, E. F. REID AND S. LEPKOVSKY 1945 Nutritional studies on milk fat. II. The growth of young rats fed glyceride fractions separated from milk fat. Ibid., *30:* 175.

MACKENZIE, C. G., J. B. MACKENZIE AND E. V. MCCOLLUM 1939 Growth and reproduction on a low fat diet. Biochem. J., *33:* 935.

MARTIN, G. J. 1939 Studies of fat-free diets. J. Nutrition, *17:* 127.

MCCUTCHEON, J. W. 1942 Preparation of linoleic acid. Organic Synthesis, *22:* 75.

NATH, E., V. H. BARKI, C. A. ELVEHJEM AND E. B. HART 1948a Further studies of the nutritive value of butterfat fractions. J. Dairy Sci., *31:* 793.

———— 1948b Studies of the alleged growth promoting property of vaccenic acid. J. Nutrition, *36:* 761.

SURE, BARNETT 1941 The existence of a new dietary factor essential for lactation. Ibid., *22:* 499.

VON EULER, B., AND H. VON EULER 1948 The functions of special higher fatty acids. Zschr. Vitamin-, Hormon- u. Fermentforsch., *1:* 474. Through Chem. Abstr., *42:* 7391.

VON EULER, B., H. VON EULER AND INEZ SABERG 1943 Nutritive factors in butter. Ernahrung, *8:* 257. Through Dairy Sci. Abstr., *6:* 126 (1944).

INTESTINAL SYNTHESIS OF NIACIN AND THE METABOLIC INTERRELATIONSHIP OF TRYPTOPHAN AND NIACIN IN THE RABBIT

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TWO FIGURES

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Ample evidence has been presented showing that several species have the ability to synthesize niacin from tryptophan. Krehl et al. ('45) found that the addition of corn to a low protein diet produced a niacin deficiency in the rat, a species which had not been shown before to require this vitamin. The animals grew very poorly, and normal growth could be restored by the addition of either niacin or tryptophan to the diet. Woolley ('45) has shown that niacin deficiency in the mouse produced by the feeding of acetylpyridine, an antivitamin for niacin, can be cured by the addition of tryptophan. Niacin deficiency has also been produced in the chick; in this species the criteria of deficiency have been poor growth, poor feathering and perosis. These symptoms respond to tryptophan therapy (Briggs, '45). Interchangeability of niacin and tryptophan has been reported for the dog (Hundley, '47; Singal et al., '48), cotton rats (Schweigert and Pearson, '48) and pigs (Luecke et al., '47).

On the other hand, it has been demonstrated that, as in the case of other water soluble vitamins, niacin is not essential for growth in the ruminants (Pearson, Schmidt and Mackey,

'39; McElroy and Goss, '39; Winegar, Pearson and Schmidt, '40; Lardinois et al., '44; and Johnson et al., '47). Apparently in this case the bacterial synthesis of niacin in the rumen of these species is large enough to meet their requirements.

The effect of niacin deficient diets on the growth of rabbits has been reported by Wooley and Sebrell ('45). These investigators found that the growth of rabbits was depressed by a niacin deficiency when they received a diet which contained 20% casein. There was no difference in the niacin content of the liver, kidney or muscle in rabbits receiving diets deficient in and supplemented with this vitamin. In a later study Wooley ('47) reported that supplementation with either niacin or tryptophan for 8 weeks produced an increase of niacin in the liver of rabbits which had been previously fed a diet low in this vitamin. There was no significant difference in the niacin content of the muscles of the group fed the tryptophan supplemented diet as compared with those of the group fed the deficient diet. The report of Wooley and Sebrell ('45) that niacin is required for normal growth in the rabbit is surprising, especially because of the work of Hundley ('47) and Schweigert and Pearson ('48), who found little or no response when niacin was fed to rats receiving diets containing 20% casein. Furthermore, rabbits synthesize large amounts of riboflavin, pantothenic acid, biotin and folic acid (Olcese, Pearson and Schweigert, '48), which probably are utilized by the animal in the course of the function of "reintestination" or "physiological coprophagy." This peculiarity of the rabbit makes this species similar to the ruminants as far as its requirement for the B vitamins is concerned, and a substantial synthesis and utilization of niacin would be expected in the rabbit. Since the results secured by Wooley did not seem to support this viewpoint, it was important to reinvestigate the need of the rabbit for niacin and to obtain more data on the niacin-tryptophan interrelationship in this species. Balance studies have been conducted in the course of this work to determine the extent of intestinal synthesis of this vitamin in the rabbit. Rate of growth and niacin and N′-methylnico-

tinamide excretion in the urine are the criteria used in interpreting the niacin-tryptophan interrelationship.

EXPERIMENTAL

Two series of experiments were conducted with New Zealand white rabbits 8 weeks old and with weights ranging from about 800 to 1,200 gm in the first experiment and from 1,000 to 1,600 gm in the second. The rabbits were housed in a room which was equipped with a heater and air cooling units. The room temperature was maintained at about 75°F. to 80°F. at all times. The rabbits were kept in groups in cages provided with wire mesh floors; water and food were given ad libitum.

TABLE 1

Composition of the diets [1]

INGREDIENTS	DIET NO.		
	1 and 2	3 and 4	5 and 6
	gm	*gm*	*gm*
Purified casein	20.0	20.0	30.0
Cerelose	58.5	58.28	48.5
Wood pulp	10.0	10.0	10.0
Tryptophan		0.22	
Salts IV [2]	3.0	3.0	3.0
A and D oil	0.5	0.5	0.5
Corn oil	8.0	8.0	8.0

[1] The diets were supplemented with the following vitamins in milligrams per kilogram: mixed tocopherols 500, choline chloride 2,000, inositol 100, 2-methyl, 1-4 naphthoquinone 0.75, pyridoxine 7, thiamine 7, riboflavin 7 and calcium pantothenate 10. Diets 2, 4 and 6 were supplemented with 600 mg of niacin per kilogram.

[2] D. M. Hegsted, R. C. Mills, C. A. Elvehjem and E. B. Hart. J. Biol. Chem., *138:* 459, 1941.

The rabbits were distributed so as to have the same number of males and females in the different groups. Otherwise the groups were formed by random selection. The composition of the diets used is shown in table 1. During the first few days of the experiment the purified diets were mixed with the stock diet to make them more acceptable to the rabbits. The addition of tryptophan to rations 3 and 4 containing

20% casein was done so as to bring the total tryptophan content of these rations to the same level as that of rations 5 and 6, which contained 30% casein.

For the metabolism studies individual metabolic cages with double bottom wire floors were used. The rabbits were placed in these cages after the 4th week on the experimental diets. In both experiments collections were made from the different groups at the same time in order to obtain data for comparable periods for all groups. The rabbits were placed in the cages for the collection of the urine and feces for a period of 72 hours. The feed consumption for the three-day period was recorded. The urine was collected in bottles containing acetic acid, and at the end of the third day the contents were mixed and the volume made up to 1,000 ml with water. Appropriate aliquots were neutralized and used for assay. The feces were dried for 24 hours at 60°C., weighed, and finely ground. Samples were then taken for determination of niacin and N′-methylnicotinamide. Niacin was determined by the microbiological method of Krehl, Strong and Elvehjem ('43) using *L. arabinosus* as the test organism and after the niacin was liberated by autoclaving the samples with H_2SO_4 for 30 min. N′-methylnicotinamide was determined in the urine by the fluorometric method of Huff, Perlzweig and Tilden ('45) without any treatment of the samples previous to the assay.

RESULTS AND DISCUSSION

The first experiment was designed to test whether rabbits fed purified diets with and without niacin would synthesize this vitamin. Ten rabbits were divided into two groups of 5 each. One group was fed diet 2, which was supplemented with 60 mg of niacin per 100 gm of diet. The other group was fed diet 1, which was not supplemented with niacin. Analysis showed that it contained 0.3 μg of niacin per gram. The rabbits were maintained on these diets for 12 weeks. During this time the rabbits in both groups made good gains; the growth data on this experiment are shown in figure 1. The rabbits fed the diet supplemented with niacin made the best

gains, with an average gain for the 12-week period of 1,637 gm. In the group fed the niacin deficient diet one rabbit died during the second week of the experiment. He had made satisfactory gains and his death is not attributed to a niacin deficiency. The other 4 rabbits in this group made an average gain of 1,057 gm in the 12-week experimental period.

The difference in the growth of the two groups of rabbits is statistically significant, showing that depression in growth resulted from the deficiency of niacin. This depression

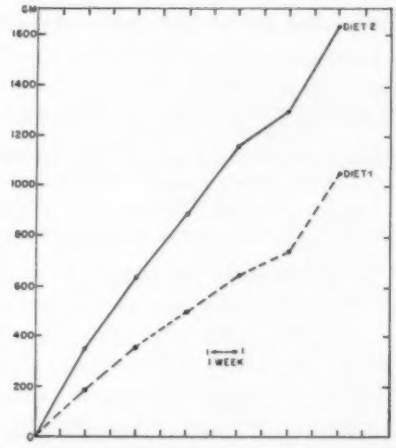

Fig. 1 Effect of niacin supplementation on the growth of rabbits. Diet 1, basal with no niacin; diet 2, basal plus niacin.

of growth is probably related to food consumption. Wooley and Sebrell ('45) found, in the course of studies with purified diets similar to the ones used in this experiment, that anorexia was the only symptom of niacin deficiency in the rabbit. In the present studies careful observations made on the rabbits fed the deficient diet failed to show any of the characteristic symptoms of niacin deficiency that have been reported for other species. Neither blacktongue nor diarrhea was present at any time. Dermatitis did not appear either, but a strong tendency of the rabbits to eat each other's hair was shown

by rabbits in both groups. This phenomenon, which had been observed before in the course of studies on B vitamin synthesis in the rabbit, is not attributed to a niacin deficiency since the symptom was shown by rabbits in both groups.

At the end of the 4th week the rabbits were started on metabolism studies. Urine and feces were collected for two different periods for each rabbit. Niacin was determined in the feed, feces and urine and N′-methylnicotinamide in the urine. A total of 8 collections was made of the urine and feces of rabbits from the niacin deficient diets and 10 collections were made of the urine and feces of rabbits receiving

TABLE 2

Ingestion and excretion of nicotinic acid by rabbits fed low and high nicotinic acid diets

(Values expressed in micrograms per 24 hours)

	LOW-NIACIN GROUP		NIACIN-SUPPLEMENTED GROUP	
Niacin ingested	11.2		25280.0	
Niacin excreted in:				
urine	139.9	(8)[1]	3059.7	(10)
feces	38.8	(8)	64.1	(10)
total	178.7		3123.8	
N′-methylnicotinamide in urine	137.8	(8)	290.0	(10)

[1] The figure in parentheses gives the number of collection periods.

the supplemented diet. The data on the excretion of niacin and N′-methylnicotinamide are shown in table 2. In the group fed the niacin deficient diet the average daily ingestion of niacin was 11.2 μg; the combined excretion in the urine and the feces was 178.7 μg of niacin per day. This 16-fold increase in the amount of niacin excreted over the amount ingested is very significant and definitely shows that substantial synthesis occurred. The fact that the fecal excretion of 38.8 μg was higher than the ingestion indicates that niacin was being synthesized in the intestine of the rabbit. This is in agreement with similar results found with this species in the case

of other B vitamins (Olcese et al., '48). The values for N′-methylnicotinamide excretion were consistently higher in the rabbits which received niacin. These animals excreted an average of 290 μg of N′-methylnicotinamide daily, as compared with 138 μg for the rabbits on the niacin deficient diet. The significance of the difference in the excretion of N′-methylnicotinamide is questionable, since in the subsequent series the values for the niacin deficient and supplemented rabbits were nearly the same.

Effect of level of tryptophan intake

This series was designed to obtain data on the conversion of tryptophan to niacin and on the growth response of rabbits fed various combinations of these supplements. Six groups of 5 rabbits each were used. They were started on diets 1, 2, 3, 4, 5 and 6. The first 4 diets contained 20% casein and were devised primarily to test the response of the rabbit to niacin and tryptophan. Rations 5 and 6 contained 30% casein and were used for the purpose of determining if tryptophan fed as casein is as effective in preventing niacin deficiency as when fed in the form of free amino acid.

The average growth of the rabbits in the different groups in this experiment is shown in figure 2. Some rabbits in the different groups refused to eat the purified diets and there was some difficulty in getting them started on the diets. The rabbits which received diet 1 showed a particular aversion for the purified diet. This accounts for the sharp decline in growth shown by these rabbits (fig. 2) at the beginning of the experiment. Since rabbits practice functional coprophagy, the question is raised of the possibility that under conditions of natural diets, which are more palatable than the purified diets, the problem of a niacin deficiency might not be so serious. It should be pointed out that the only symptom attributed by Wooley and Sebrell ('45) to niacin deficiency in rabbits fed purified diets was anorexia. It is interesting to note that these investigators found rabbits that made gains when fed the niacin deficient diets. In the present experiment there

was also one rabbit fed the deficient diet which made fairly good gains. However, the rabbits on the low niacin intake lost an average of 34 gm in 10 weeks. The rabbits which received ration 2 with 20% casein and niacin gained an average of 680 gm in 10 weeks. The rabbits which received ration 3 with 20% casein and tryptophan gained 864 gm. The rabbits which received ration 4 with 20% casein plus niacin and tryptophan gained 693 gm. The rabbits receiving ration 5 with

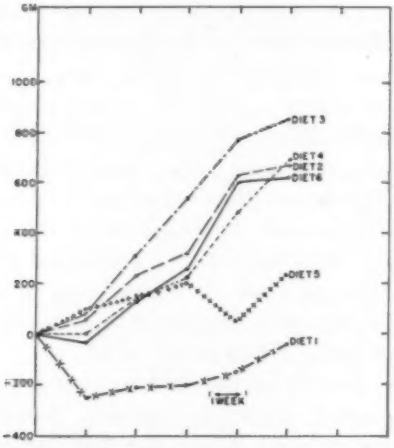

Fig. 2 Effect of different diets on the growth of rabbits. Diet 1, basal with 20% casein and no niacin; diet 2, basal plus niacin; diet 3, basal plus tryptophan; diet 4, basal plus niacin and tryptophan; diet 5, 30% casein without niacin; and diet 6, 30% casein plus niacin.

30% casein but no niacin or tryptophan made an average gain of only 247 gm, while the rabbits receiving ration 6 with 30% casein plus niacin gained 629 gm.

Comparing the growth of the rabbits which received casein without added nicotinic acid with that of those receiving 20% casein plus tryptophan at the same level as the 30% casein group, it can be seen that the gains in weight of the group receiving the free amino acid were much higher than in the case of the rabbits receiving the extra tryptophan from casein.

This seems to support the finding of Rosen et al. ('46) that while tryptophan, administered as the free amino acid, may serve as an excellent source of niacin, tryptophan ingested as protein is considerably less effective.

Several rabbits in the different groups died. One rabbit receiving the niacin deficient diet and one receiving the tryptophan supplemented diet died the first week of the experiment. The three rabbits that refused to eat in the group fed the 20% casein basal diet died from the 7th to the 10th week. During the 8th week one rabbit in the group fed 20% casein plus niacin developed paraplegia and was killed. Two rabbits

TABLE 3

Urinary excretion of niacin and N'-methylnicotinamide by rabbits

DIET NO.	TREATMENT DIETARY	INGESTION OF		EXCRETION IN URINE	
		Niacin	Tryptophan	Niacin	N'-methyl-nicotinamide
		μg/24 hr.	*mg/24 hr.*	*μg/24 hr.*	*μg/24 hr.*
1	Basal (20% cas.)	12.1	177.8	75.6	552.3
2	Basal + niacin	24,180.0	177.2	1,911.3	528.0
3	Basal + trypto.	10.3	226.8	176.5	758.9
4	Basal + niacin + trypto.	31,080.0	341.8	2,596.3	663.5
5	30% casein	9.9	218.3	167.3	818.1
6	30% casein + niacin	23,100.0	254.0	1,716.3	710.3

fed 30% casein died during the 5th week of the experiment and one fed 30% casein plus niacin died in the 6th week.

Studies on the urinary excretion of niacin and N'-methylnicotinamide were conducted starting the 6th week. The average values obtained in these studies are shown in table 3. The urinary excretion of niacin by the rabbits in the group receiving the basal diet was 75.6 μg per day. The rabbits which received a diet supplemented with tryptophan and those which received a diet with 30% casein excreted in the urine an average of 176.5 and 167.3 μg of niacin per day, respectively. This 2.5-fold increase in excretion by the rabbits on

the basal diet shows that an increased synthesis of niacin resulted from the feeding of extra tryptophan. The fact that the basal diet contains 0.66% as much tryptophan as diets 3 and 5 indicates that the tryptophan is first used by the rabbit to meet its requirements for this amino acid and that the synthesis of niacin occurs at the maximum rate only after the requirements for tryptophan per se are met. This suggests that the conversion of tryptophan to niacin occurs within the tissues of the animal itself and is not due to increased intestinal synthesis, since if the latter were the case it is difficult to explain how an increase of 33% in the tryptophan in the diet would bring a 2.5-fold increase in the excretion of niacin.

The rabbits fed diets 2, 4 and 6, which were supplemented with niacin, excreted much higher amounts of niacin through the urine than the other animals but still only a small fraction of the total amount ingested.

The values for the urinary excretion of N′-methylnicotinamide in this experiment are shown in table 3. The average daily excretion of N′-methylnicotinamide in the urine of the rabbits fed the low niacin and 20% casein diet was 552.3 μg as compared with 528 μg for the rabbits fed a diet containing 20% casein plus niacin. There was considerable variation within a group and the differences between these two groups are not considered significant. The results secured in this case are in agreement with the finding of Huff and Perlzweig ('43) that there is no increase in the urinary excretion of N′-methylnicotinamide after the feeding of niacin to the rabbit. The other 4 groups of rabbits receiving additional tryptophan, either as free amino acid or higher levels of casein, excreted in the urine larger amounts of N′-methylnicotinamide than the two previous groups. The rabbits fed 20% casein plus tryptophan excreted 758.9 μg of N′-methylnicotinamide, as compared with 663.5 μg by the rabbits fed 20% casein plus niacin and tryptophan. The rabbits receiving the 30% casein diets without and with casein excreted 818.1 μg and 710.3 μg of N′-methylnicotinamide, respectively. On the whole no great variations among the groups were found.

The data presented in this paper show that niacin is synthesized by the rabbit by both microbial activity in the digestive tract and metabolic processes within the tissues. The fact that tryptophan fed as the free amino acid gave a better growth response than when fed in the form of casein may be taken as an indication that free tryptophan is superior for synthesis of nicotinic acid than that found in protein. However, it may also be that the increased rate of protein metabolism increases the demand for niacin. The administration of an unbalanced protein in this case would decrease the efficiency of tryptophan as a source of niacin.

SUMMARY

1. The feeding of a niacin deficient diet to rabbits resulted in a decreased gain in weight. One group of rabbits fed a purified diet supplemented with 60 mg of niacin per 100 gm of diet showed an average gain in weight of 1,637 gm, as compared to a gain of 1,057 gm made by a group that received a similar diet without the addition of niacin.

2. Balance studies showed that considerable amounts of niacin were being synthesized by the rabbits fed the low niacin diet. The average daily ingestion of niacin for this group was 11.2 μg, with a corresponding niacin excretion of 178.7 μg. The average daily fecal excretion of niacin amounted to 38.8 μg, showing that synthesis was taking place in the digestive tract.

3. Growth data secured with 6 groups of rabbits fed different levels of niacin and fed tryptophan as the free amino acid and as additional protein showed that niacin could be synthesized from its precursor tryptophan. One group of rabbits receiving the basal diet with 20% casein but no additional tryptophan or niacin lost an average of 34 gm in 10 weeks. The group receiving the basal diet supplemented with niacin gained an average of 680 gm in the same period. Rabbits receiving the basal ration plus tryptophan gained 864 gm, and the rabbits in the control group receiving the basal

diet supplemented with both niacin and tryptophan gained 693 gm.

4. Tryptophan fed as protein was not as effective as when fed as free amino acid, as evidenced by the fact that the rabbits in a group fed additional tryptophan in the form of casein gained only 247 gm as compared to the 864 gm gained by the group which was fed the same amount of tryptophan in the form of free amino acid.

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LITERATURE CITED

BRIGGS, G. M. 1945 Influence of gelatin and tryptophan on nicotinic acid requirements of chicks. J. Biol. Chem., *161:* 749.

HUFF, J. W., AND W. A. PERLZWEIG 1943 The probable identity of Najjar and Holt's fluorescent substance, F_2. Science, *97:* 538.

HUFF, J. W., W. A. PERLZWEIG AND M. W. TILDEN 1945 A simple method for the determination of N'-methylnicotinamide in urine. Fed. Proc., *4:* 92.

HUNDLEY, J. M. 1947 Production of niacin deficiency in rats. J. Nutrition, *34:* 253.

JOHNSON, B. C., A. C. WIESE, H. H. MITCHELL AND W. B. NEVENS 1947 The metabolism of nicotinic acid and its role in the nutrition of the calf. J. Biol. Chem., *167:* 729.

KREHL, W. A., F. M. STRONG AND C. A. ELVEHJEM 1943 Determination of nicotinic acid. Modifications in the microbiological method. Ind. Eng. Chem., Anal. Ed., *15:* 471.

KREHL, W. A., L. J. TEPLY AND C. A. ELVEHJEM 1945 Growth retarding effect of corn in nicotinic acid low rations and its counteraction by tryptophan. Science, *101:* 489.

LARDINOIS, C. C., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1944 Rumen synthesis of the vitamin B complex as influenced by ration composition. J. Dairy Sci., *27:* 579.

LUECKE, R. W., W. M. MCMILLEN, F. THORP, JR. AND C. TULL 1947 The relationship of nicotinic acid, tryptophan and protein in the nutrition of the pig. J. Nutrition, *33:* 251.

MCELROY, L. W., AND HAROLD GOSS 1939 Report on 4 members of the vitamin B complex synthesized in the rumen of the sheep. J. Biol. Chem., *130:* 437.

OLCESE, ORLANDO, P. B. PEARSON AND B. S. SCHWEIGERT 1948 The synthesis of certain B vitamins by the rabbit. J. Nutrition, *35:* 577.

PEARSON, P. B., H. SCHMIDT AND A. K. MACKEY 1939 Effect of pellagra-producing diet on herbivora. Proc. Soc. Exp. Biol. Med., *40:* 423.

ROSEN, F., J. W. HUFF AND W. A. PERLZWEIG 1946 The effect of tryptophan on the synthesis of nicotinic acid in the rat. J. Biol. Chem., *163:* 343.

SCHWEIGERT, B. S., AND P. B. PEARSON 1948 Further studies on the metabolism of tryptophan and nicotinic acid by the rat and other animals. Ibid., *172:* 485.

SINGAL, S. A., V. P. SYDENSTRICKER AND J. M. LITTLEJOHN 1948 The role of tryptophan in the nutrition of dogs on nicotinic acid-deficient diets. Ibid., *176:* 1051.

WINEGAR, A. H., P. B. PEARSON AND H. SCHMIDT 1940 The synthesis of nicotinic acid in the body of the sheep. Science, *91:* 508.

WOOLEY, J. G. 1947 Niacin deficiency in rabbits and response to tryptophan and to niacin. Proc. Soc. Exp. Biol. Med., *65:* 315.

WOOLEY, J. G., AND W. H. SEBRELL 1945 Niacin (nicotinic acid) an essential growth factor for rabbits fed a purified diet. J. Nutrition, *29:* 191.

WOOLLEY, D. W. 1945 Production of nicotinic acid deficiency with three-acetyl-pyridine, the ketone analogue of nicotinic acid. J. Biol. Chem., *157:* 445.

URINARY PANTOTHENATE, BLOOD GLUCOSE, AND INORGANIC SERUM PHOSPHATE IN PATIENTS WITH METABOLIC DISORDERS TREATED WITH DOSES OF PANTOTHENATE [1]

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INTRODUCTION

Evidence has accumulated that pantothenic acid participates at some stage in the metabolism of carbohydrate. Pilgrim et al. ('42) and Pilgrim and Elvehjem ('44) found a decreased rate of oxygen consumption in the presence of pyruvate by liver homogenates of pantothenic acid deficient rats. Recent investigations have shown that coenzyme A, which is involved in the utilization of acetate and possibly in the breakdown of pyruvate to acetate, contains large amounts of pantothenic acid (Lipmann et al., '47; Novelli and Lipmann, '47; Kaplan and Lipmann, '48). Animals on a pantothenic acid deficient diet for two to three weeks show a gradual depletion of coenzyme A in their tissues (Olson and Kaplan, '48), an inability to acetylate injected *p*-aminobenzoic acid (PABA) (Riggs and Hegsted, '48), and adrenal cortical damage if the diet is continued long enough (Daft and Sebrell, '39). In spite of the many reports on the role of

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pantothenic acid in animals, its role in human subjects has not as yet been demonstrated.

This study is concerned with an attempt to evaluate the role of pantothenic acid in patients with diseases of metabolism involving the carbohydrate cycle. The patients studied had either diabetes, Addison's disease, or cirrhosis of the liver and, in addition, two patients with chromophobic tumor of the pituitary were studied. Amounts of pantothenate far above the presumed daily requirement were given to the subjects in the form of either calcium pantothenate or panthenol. The daily dose was from 12 to 20 gm, one-third at each meal.

EXPERIMENTAL

Whole blood was collected in vials containing oxalate and precipitated immediately. Blood sugar was determined on the filtrate by the Nelson modification of the Somogyi method (Nelson, '45). Inorganic phosphate was determined on serum by the Fiske and Subbarow ('25) method modified for the Coleman colorimeter. Pantothenic acid was determined in urine by the methods of Atkin et al. ('44) and Skeggs and Wright ('44).

The first observations were concerned with the excretion of pantothenic acid by the subjects. Urine samples were collected for 24 hours while the patients were on their regular diets. The diets were then supplemented with 12 to 20 gm of pantothenate daily as indicated. On normal diets the excretion of pantothenic acid ranges from 2.1 mg to 5.0 mg, with an average of 3.7 mg (Rubin et al., '48; Sarett, '45). The 24-hour excretion of pantothenic acid by the subjects in this study is shown in table 1. The range was from 2.6 mg to 8.6 mg per 24 hours. The latter figure, in a patient with cirrhosis of the liver, may reflect the inclusion in the diet of vitamin supplements of which we had no knowledge. The administration of panthenol, in equivalent doses of calcium pantothenate, was associated with a greater urinary excretion of pantothenate, as is shown in the table. The same results have been reported by Rubin et al. ('48).

TABLE 1

The 24-hour excretion of pantothenate

CASE	DIAGNOSIS	DIET	DAILY DOSE CALCIUM PANTOTHENATE OR "PANTHENOL"	CA.PAN. EXCRETED	% DOSE EXCRETED	REMARKS
				mg/24 hr.		
MA	Addison's disease	Hospital	None	4.7	...	Average of 3 urine samples 24 hr. each
MA	Addison's disease	Hospital	20 gm Ca Pan.	2,900.0	14.4	Average of 3 urine samples 24 hr. each
MA	Addison's disease	Hospital	17 gm Panthenol	12,500.0	62.4	Average of 5 urine samples 24 hr. each
GA	Addison's disease	Hospital	None	4.0	...	
GA	Addison's disease	Hospital	12 gm Ca Pan.	2,100.0	17.5	Calculated on basis of mg/ml; 24-hr. urine samples not available
HI	Chromophobic tumor	Hospital	None	6.0	...	
NE	of pituitary	Hospital	15 gm Ca Pan.	1,400.0	9.0	Average of 4 urine samples 24 hr. each
LO	Cirrhosis of liver	Hospital	None	6.9	...	Average of 3 urine samples 24 hr. each
GR	Cirrhosis of liver	Hospital	None	2.6	...	
GR	Cirrhosis of liver	Hospital	15 gm Panthenol	2,800.0	...	12-hr. collection (overnight)
GR	Cirrhosis of liver	Hospital	15 gm Panthenol	4,400.0	...	12-hr. collection (overnight)
VA	Cirrhosis of liver	Hospital	None	8.6	...	Average of 2 urine samples 24 hr. each
VO	Diabetes mellitus	Home diet	None	4.2	...	On 8 units of insulin daily

TABLE 2

Excretion of urinary pantothenate after glucose or insulin administered intravenously

CASE	DIAGNOSIS	THERAPY	URINARY EXCRETION OF CALCIUM PANTOTHENATE				
			Before glucose		After glucose		
			Urine Vol.	Ca Pan.	Urine Vol.	Ca Pan.	
			ml	*mg/ml*	*ml*	*mg/ml*	
GA	Addison's disease	None	575	0.0026	150	0.0033	
GA	Addison's disease	Ca Pan. 12,000 mg/day	178	0.1400	210	0.1400	
MA	Addison's disease	"Cortin" and Doca [1]	2000 [2]	0.0020	310	0.0020	
MA	Addison's disease	Doca [1]	1630 [2]	0.0030	288	0.0030	
MA	Addison's disease	Ca Pan. 15,000 mg/day	800	0.8800	365	0.6000	
NE	Pituitary tumor	None	80	0.0130	190	0.0140	
HI	Pituitary tumor	None	1080	0.0060	246	0.0040	
AU	Cirrhosis of liver	"Liver extract" [3]	88	0.0110	246	0.0070	
HO	Cirrhosis of liver	"Liver extract" [3]	5	0.0400	27	0.0300	
LO	Cirrhosis of liver	"Liver extract" [3]	665	0.0040	120	0.0070	
DA	Diabetes mellitus	Diet and insulin	190	0.0090	246	0.0060	
			Before insulin		*After insulin*		*Units insulin I.V.*
VO	Diabetes mellitus	Diet and insulin	920	0.0047	150	0.0077	16
OR	Diabetes mellitus	Diet	40	0.0047	115	0.0046	20

[1] Desoxycorticosterone acetate.
[2] Twenty-four-hour urines collected up until beginning of test.
[3] This liver extract contained 191 μg/ml of pantothenic acid.

In the second part of the study the effect of the intravenous administration of glucose or insulin on the urinary excretion of pantothenic acid was observed. All the tests were done on patients fasted overnight. Pantothenic acid was measured in the urine voided just before the infusion and in the urine collected during three to 4 hours after the injection. Occasionally an overnight or 12-hour specimen was used for the

TABLE 3

Blood glucose and phosphate changes during intravenous glucose tolerance test in two patients with Addison's disease, before and after administration of pantothenate

	HOURS AFTER GLUCOSE	BEFORE PANTOTHENATE				AFTER PANTOTHENATE			
		Blood glucose		Inorganic PO_4 (Serum)		Blood glucose		Inorganic PO_4 (Serum)	
		mg %		mg %		mg %		mg %	
CASE MA, MALE	Fasting	80 [1]	72 [2]	3.22 [1]	3.42 [2]	75 [3]		3.42 [3]	
	½	..	210	3.00	3.20	248		3.28	
	1	140	148	3.06	3.12	165		3.37	
	2	61	75	3.08	3.24	85		348	
	3	60	50	3.00	3.40	58		3.73	
	4	59	62	3.14	3.52	60		3.80	
CASE GA, FEMALE	Fasting	68 [4]		3.86 [4]		78 [5]	70 [6]	4.00 [5]	4.04 [6]
	½	176		3.58		254	180	3.72	3.78
	1	126		3.72		159	140	3.54	3.78
	2	81		3.74		94	88	3.63	3.90
	3	72		3.74		74	72	3.67	3.83

[1] Receiving 5 ml cortical extract, 2.5 mg DOCA (desoxycorticosterone acetate) and 15 gm salt daily.

[2] No cortical extract for 13 days; receiving 2.5 mg DOCA and 15 gm salt daily.

[3] Receiving 5 ml cortical extract, DOCA, salt, and calcium pantothenate (15 gm for 26 days) daily.

[4] Receiviing 20 ml cortical extract, 5 mg DOCA, and salt daily.

[5] Receiving 20 ml cortical extract, DOCA, and salt daily; and calcium pantothenate, 12 gm daily for 7 days.

[6] No cortical extract for 7 days; 5 mg DOCA and salt daily; calcium pantothenate, 12 gm daily for 20 days.

pre-injection assay. Glucose was administered as a 20% solution infused over a period of 30 minutes, the dose being arranged so that the patient received 0.5 gm per kilogram of body weight. Observations under these conditions were made on the patients with chromophobic tumors of the pituitary,

TABLE 4

Blood glucose and phosphate levels during intravenous administration of glucose or insulin

(Levels were determined before and again after the administration of large doses of calcium pantothenate)

	HOURS AFTER GLUCOSE OR INSULIN	BEFORE PANTOTHENATE		AFTER PANTOTHENATE	
		Blood glucose	Inorganic PO_4 (Serum)	Blood glucose	Inorganic PO_4 (Serum)
		mg %	mg %	mg %	mg %
GLUCOSE I.V.	Fasting	88	2.65	91 [1]	2.78 [1]
	½	270	2.25	246	2.52
	1	131	2.22	102	2.38
	2	58	2.40	68	2.47
	3	76	2.48	79	2.46
INSULIN I.V.	Fasting	232 [2]	2.72 [2]	213 [3]	3.28 [3]
	½	97	2.04	143	2.74
	1	70	2.50	97	2.76
	2	103	2.80	85	2.86
	3	125	3.48	93	3.34

[1] Patient had received 12 gm of calcium pantothenate daily for 5 days before test.

[2] Patient received 16 units of insulin intravenously for test.

[3] Test repeated 26 days later with 16 units of insulin intravenously; 12 gm calcium pantothenate daily for 7 days prior to test.

on the patients with Addison's disease, on three patients with diabetes, and on three patients with cirrhosis of the liver. The results of administering glucose intravenously are shown in table 2. The excretion of pantothenate was not influenced by raising the blood sugar level. Similarly, the effect of accelerating carbohydrate metabolism by administering insulin

intravenously had no influence on the excretion of pantothenate, as is also shown in table 2.

Table 3 gives the glucose and inorganic phosphate levels during the administration of glucose in the two patients with Addison's disease. The administration of pantothenic acid for varying periods prior to the glucose tolerance test did not affect the fasting levels of glucose or phosphate or alter the nature of the curves. The same type of experiments were done on the patients with chromophobic tumors of the pituitary and on the diabetic patients. Examples of the results of such experiments are given in table 4. Again pantothenate had no appreciable effect on the levels of glucose or inorganic phosphate following the intravenous administration of glucose, nor was the effect of insulin given intravenously modified by the administration of pantothenate.

DISCUSSION

The results indicate that the urinary excretion of pantothenic acid is normal in patients with the diseases of metabolism that were included in the group studied. When calcium pantothenate or panthenol was added to the diet, the excretion of pantothenic acid was increased significantly. These findings are the same as were observed in normal subjects by Rubin et al. ('48, '49), and fit in with the observations of Wright et al. ('46) with respect to the renal clearance of pantothenic acid. These authors showed that at ordinary normal plasma concentrations only a trace of pantothenic acid is excreted in the urine, but that on increasing the plasma concentration of the compound by oral or intravenous administration there is an abrupt rise in the clearance of pantothenic acid.

Wright ('42) showed that when glucose was administered to rabbits the blood level of pantothenic acid was decreased, presumably as a result of increasing carbohydrate utilization. In the observations by the present authors the levels of blood pantothenate were not determined, but in these short-term experiments no significant effect on the excretion of

pantothenic acid was observed as a result of glucose or insulin administration.

The fall in serum inorganic phosphate that occurs after the administration of glucose or insulin is probably due to the formation of an intermediate hexosphosphate (Hartman and Bolliger, '25) or to the diffusion of glucose into the tissues with the formation of a phosphate compound (Levine et al., '49). Our results indicate that the administration of excessive doses of pantothenic acid for periods of 5 to 26 days does not influence carbohydrate metabolism as reflected by the blood glucose or phosphate levels following the administration of either glucose or insulin intravenously. It should be borne in mind that no experiments were done on pantothenic acid deficient diets, but that the experiments were conducted while the patients were on normal diets and when these diets were supplemented with excessive amounts of pantothenic acid.

The administration of very large doses of pantothenic acid did not affect the blood glucose levels in the patients with diabetes nor was there any change in the insulin requirement during the period of pantothenic acid administration. This observation is in agreement with the finding (Irwin and Ralli, '48) that in alloxan diabetic rats neither the addition nor absence of pantothenic acid in the diet had any effect on the level of blood sugar or on the urinary excretion of glucose.

SUMMARY

The daily excretion of pantothenic acid was studied in patients with diabetes, Addison's disease, cirrhosis of the liver, and chromophobic tumor of the pituitary. On a normal diet the excretion of pantothenic acid in all subjects varied from 2.6 to 8.6 mg per 24 hours. When calcium pantothenate was added to the diet the daily excretion rose so that at least 9% of the ingested pantothenate was excreted. When panthenol was the source of pantothenate, the excretion of pantothenic acid was still further increased. Insulin and glucose given intravenously did not affect the excretion of pantothenic

acid in any of the patients studied. The blood levels of glucose and inorganic phosphate, determined following the intravenous administration of glucose or insulin, were not influenced by large daily doses of calcium pantothenate.

LITERATURE CITED

ATKIN, L., W. L. WILLIAMS, A. S. SCHULTZ AND C. N. FREY 1944 Yeast microbiological methods for determination of vitamins. Ind. Eng. Chem., Anal. Ed., *16:* 67.

DAFT, F. S., AND W. H. SEBRELL 1939 Hemorrhagic adrenal necrosis in rats on deficient diets. Pub. Health Rep., Wash., *54:* 2247.

FISKE, C. H., AND Y. SUBBAROW 1925 Colorimetric determination of phosphorus. J. Biol. Chem., *66:* 375.

HARTMAN, F. W., AND A. BOLLIGER 1925 Curve of inorganic blood phosphates during the sugar tolerance test. J. Am. Med. Assoc., *85:* 653.

IRWIN, L., AND E. P. RALLI 1948 Pantothenic acid and carbohydrate metabolism in the rat. Am. J. Physiol., *153:* 1948.

KAPLAN, N. O., AND F. LIPMANN 1948 The assay and distribution of coenzyme A. J. Biol. Chem., *175:* 515.

LEVINE, R., S. D. LOUBE AND H. F. WEISBERG 1949 Nature of the action of insulin on the level of serum inorganic phosphate. Fed. Proc., *8:* 95.

LIPMANN, F., N. O. KAPLAN, G. D. NOVELLI, L. C. TUTTLE AND B. M. GUIRARD 1947 Coenzyme for acetylation, a pantothenic acid derivative. J. Biol. Chem., *167:* 869.

NELSON, N. 1945 A photometric adaptation of the Somogyi method for the determination of glucose. Ibid., *160:* 69.

NOVELLI, G. D., AND F. LIPMANN 1947 Bacterial conversion of pantothenic acid to coenzyme A (acetylation) and its relation to pyruvic oxidation. Arch. Biochem., *14:* 23.

OLSEN, R. E., AND N. O. KAPLAN 1948 The effect of pantothenic acid deficiency upon the coenzyme A content and pyruvate utilization of rat and duck tissues. J. Biol. Chem., *175:* 515.

PILGRIM, F. J., A. E. AXELROD AND C. A. ELVEHJEM 1942 The metabolism of pyruvate by liver from pantothenic acid- and biotin-deficient rats. Ibid., *145:* 237.

PILGRIM, F. J., AND C. A. ELVEHJEM 1944 Further studies of pyruvate metabolism by livers from vitamin B-deficient rats. Ibid., *156:* 257.

RIGGS, T. R., AND D. M. HEGSTED 1948 The effect of pantothenic acid deficiency on acetylation in rats. Ibid., *172:* 539.

RUBIN, S. H., J. M. COOPERMAN, M. E. MOORE AND J. SCHEINER 1948 The physiological availability of pantothenyl alcohol. J. Nutrition, *35:* 499.

RUBIN, S. H., L. DREKTER, M. E. MOORE AND R. PANKOPF 1949 Availability of panthenol and calcium panthothenate in relation to food intake. Fed. Proc., *8:* 393.

SARETT, H. P. 1945 The metabolism of pantothenic acid and its lactone moiety in man. J. Biol. Chem., *159:* 321.

SKEGGS, H. R., AND L. D. WRIGHT 1944 The use of Lactobacillus arabinosis in the microbiological determination of pantothenic acid. Ibid., *156:* 21.

WRIGHT, L. D. 1942 The effect of glucose administration on the level of blood pantothenic acid. Ibid., *142:* 445.

WRIGHT, L. D., K. H. BEYER, H. R. SKEGGS, H. F. RUSSO AND E. A. PATCH 1946 The renal clearance of pantothenic acid. Am. J. Physiol., *145:* 633.

HYPERVITAMINOSIS A IN THE DOG

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SIX FIGURES

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The production of hypervitaminosis A in the rat, the mouse and the guinea pig, with concurrent bony changes and toxic symptoms, has been reported by numerous workers—Takahashi et al. ('25), Harris and Moore ('28), Collazo, Rubino and Varela ('29), Bomskov and Seemann ('33), Collazo and Rodriguez ('33), Drigalski ('33), Moll et al. ('33), Davies and Moore ('34), Strauss ('34–'35), Weslaw et al. ('38), Cornil, Chevallier and Paillas ('39), Noetzel ('39), Wolbach and Bessey ('42), Moore and Wang ('45), Wolbach ('47). It has been recognized as a result of accidental overdosage in man (Rodahl and Moore, '43; Josephs, '44; and Rothman and Leon, '48). The dog, however, has never been studied from this viewpoint. Corroboration in a new species of the findings relating to hypervitaminosis A, and the convenience of using a larger animal because of the need of further work on the chemical aspects of the problem, have prompted the present investigation.

EXPERIMENTAL

A litter of 5 greyhound puppies born of thoroughbred greyhounds, laboratory animals of several years' standing,

was selected. These puppies were two months old and in good health when the experiment was begun. They had been with the mother until a week before the start of the experimental period. At this time they were transferred to separate cages and put on a diet of milk, bread, horsemeat, dog chow and occasional vitamin D supplement in the form of cod liver oil. Their weights at this time ranged between 4.5 and 5.5 kg. The distribution between the sexes was three males and two females. One of each sex, both of them representing the heavier dogs of the litter and weighing 5 + kg, was selected for excessive vitamin A administration.

Dosage and manner of administration

Vitamin A in the form of concentrate [1] with a potency of 500,000 I.U. per gram was used. This was fed at a level of 300,000 I.U. per kilogram of body weight and was given daily, Sundays excepted. It was administered by mouth for the first 22 days of the experiment. Since the anticipated result was not obtained after this period, it was thought wiser to discontinue the oral route of administration. The dogs were then fed the concentrate by stomach tube and this was followed by either 4.0 ml of corn oil or of olive oil, in order to rinse the vitamin preparation down the tube. No complications in tube-feeding the dogs were encountered.

Two different samples of concentrate, with similar potencies, were employed. Both of them had been repeatedly used on guinea pigs and when fed at the above-mentioned level were known to give a typical picture of vitamin A excess, with fracture of long bones, after a 12- to 16-day period.

Procedures followed during the course of the experiment

The dogs were weighed daily, Sundays excepted. X-rays to determine bony changes were taken of the experimental animals and the controls at the beginning of the experiment

[1] Purchased from Distlillation Products, Inc., Rochester, N. Y.

and at intervals thereafter. The clinical course was carefully followed.

The dogs were bled at frequent intervals and except for some of the earlier bloods, which were studied merely for their cholesterol and phospholipid content, determinations were done on specimens obtained after a 12-hour fast. The dogs were followed from the standpoint of serum cholesterol and serum lipid-phosphorus throughout the period under observation. Serum inorganic phosphorus, serum alkaline phosphatase and serum vitamin A levels were followed from the 45th day on. Studies on blood sugar, non-protein nitrogen, uric acid, creatinine, serum sodium and serum chloride were done during the final stages of the experiment. The bromsulphonphthalein test was done on both experimental animals, using normals as controls, once the syndrome was well established.

Serum vitamin A levels were determined by the Lowry-Bessey method (Lowry and Bessey, '45), checked occasionally by the May method (May et al., '40). An unpublished Fiske and SubbaRow method for lipid-P was employed. Cholesterol was determined by the Lieberman-Burchard (Bloor, Pelkan and Allen, '22; Mirsky and Bruger, '32) colorimetric procedure. The Folin-Malmros (Folin and Malmros, '29) micromethod was used for blood sugars. Non-protein nitrogen, uric acid, creatinine and creatine were measured by the routine Folin procedures (Folin, '34). Bodansky's method (Bodansky, '32) for determining serum alkaline phosphatase and serum inorganic phosphorus was utilized. Serum Ca was determined by the Fiske and Adams method (Fiske and Adams, '31), serum chloride by the Whitehorn method (Whitehorn, '20–'21), and serum sodium by Butler and Tuthill's (Butler and Tuthill, '31) method. Bromsulphonphthalein retention, using a 5% solution and administering 5 mg of dye per kilogram of body weight, was measured at the end of 5-, 15-, and 30-minute periods.

RESULTS

Weight changes

The male controls showed a satisfactory weight gain throughout. During the 67-day period under observation they gained on the average 135 and 148 gm a day, respectively. The female control during the first 56 days gained an average of 93 gm daily. A sudden drop then occurred during the next 6 days due to an attack of diarrhea of unexplained origin, followed by a slower return to normal. Comparing the experimental male and female with their respective male and female controls up to the point where a drop in the weights

TABLE 1

Daily weight gain in grams — 53-day period

	CONTROL		EXPERIMENTAL
	Dog 1	Dog 3	Dog 2
Male	160	168	93
	Dog 5		Dog 4
Female	111		73

of the experimental animals took place (53rd day), the average daily weight gains are as indicated in table 1. From the 53rd day on, both experimental animals showed a precipitous fall in weight, the male losing 23% of his maximum weight in 8 days, the female 15% in 5 days.

Clinical course

The dogs seemed well and normally active up to and beyond the point where the decline in weight first began to manifest itself. On the 30th day of the experiment it was first noted that their appetites were failing. Solid food, especially meat, was refused either wholly or in part. Except for this they continued to react normally until two to three days after the rather precipitous fall in weight. From then on, both rapidly declined.

The female animal, which seemed to respond more emphatically and promptly to the excessive vitamin A, refused to stand, yelped when touched, and shivered more or less continuously. She acted as though she were in great discomfort. Twenty-four hours later her buttocks were soiled, she smelled strongly of urine, licked her front paws more or less continuously as does a dog which has sustained an injury to them, and was unable to use her hind legs. No fractures could be detected but there was extreme tenderness over all epiphyses. During the last day of life this animal seemed relatively indifferent to her surroundings. She was sacrificed under intravenous pentobarbital anesthesia on the 58th day of the experiment.

The male dog (no. 2), which was maintained on the regime for a longer period of time and was sacrificed on the 69th day under pentobarbital anesthesia, differed from the female in that he showed the effects of a greater weight loss. Skeletal landmarks seemed unusually prominent. The normal dorsal curvature of the spine was exaggerated. Movements of the shoulders would bring the medial borders of the scapulae into profile, as though there were no force to counteract the pull of such muscles around the shoulder joint as had scapular attachments. Bony protuberances, bony ridges and joints seemed more prominent because of excessive and rapid wasting. Hyperesthesia of the skin and extreme tenderness over all extremities were marked in the last few days of life. Unwillingness to stand was a symptom occurring during the last 48 hours, but at no time were gross fractures detectable. Both in this dog and the female a moderate degree of exophthalmos was noted during the last 7 to 10 days of life, but no other ophthalmological changes were noted in either dog.

X-ray findings

Satisfactory x-ray films of a few bones only were obtained at the conclusion of the experiment. The following description (dog 2) is based on the comparison with x-ray films from control dog 1.

The epiphyseal lines of all the long bones of the experimental dog are markedly narrower than those of control bones. The cortices of the femur, tibia, radius and ulna of the experimental dog are less dense and thinner. The most striking change is shown in the fibula, which is markedly reduced in width and density, almost to complete disappearance a few centimeters be-

low the proximal end, whereas the cartilaginous end is of approximately normal width. The tibia shows marked loss of density of the crest just below the epiphysis of the tuberosity and in this location the outline of the bone is fuzzy in appearance. The distal ends of the ulna are markedly expanded, fuzzy, and, in dog 2, the appearance suggests that of an impacted fracture.

The distal end of the radius shows narrowing, as compared to that of the control, and the periosteal borders for 2 cm proximal to the epiphyseal line are irregular and fuzzzy in outline.

Blood chemistry

Aside from the changes in cholesterol and lipid-phosphorus values, there was little that was remarkable in the chemical blood picture. The non-protein nitrogen rose terminally to a very moderate degree. There was a terminal increase in blood creatine, probably a reflection of excessive muscle wasting. Dog 4 showed an increase in serum calcium. Phosphatase and inorganic phosphorus values lay within normal limits. A terminal and fairly abrupt rise in serum sodium (153 milliequivalents per liter of serum) was present in dog 4. There was little difference between experimental animals and controls as regards bromsulphonphthalein retention, though a slightly increased retention in the hyper A animals was seen, an observation also noted in guinea pigs (Maddock, '42). Serum vitamin A levels, remarkably high normally in the dog (Clausen et al., '40; Wakerlin and Moss, '43), showed an 8- to 40-fold increase (table 2).

Cholesterol and lipid phosphorus values remained unaltered in both experimental animals until symptoms of acute hypervitaminosis A were well established. The female, three days before her exitus, showed a moderate decline in both of these values. This was followed on the final day by a secondary rise which, however, fell short of the previous normal levels. In the male the drop in both cholesterol and lipid-P was more abrupt, and these values remained at a low level until the animal was sacrificed (table 2).

TABLE 2

Vitamin A, cholesterol and lipid-P

DAY OF EXPERIMENT	HYPER A DOGS						CONTROL DOGS								
	Dog 2			Dog 4			Dog 1			Dog 3			Dog 5		
	Vitamin A	Cholesterol	Lipid-P	Vitamin A	Cholesterol	Lipid-P	Vitamin A	Cholesterol	Lipid-P	Vitamin A	Cholesterol	Lipid-P	Vitamin A	Cholesterol	Lipid-P
	I.U.%	*mg %*	*mg %*	*I.U.%*	*mg %*	*mg %*	*I.U.%*	*mg %*	*mg %*	*I.U.%*	*mg %*	*mg %*	*I.U.%*	*mg %*	*mg %*
1					235.0	18.23									
2		230.0	16.15												
3								245.0							
4														155.0	15.55
16		255.0	18.39								188.0	15.02			
25		226.0	18.42												
26					230.0	21.35		235.0	20.2		230.0	23.93			
32		219.0	18.50												
33					241.0	18.20								202.0	16.10
40		203.0			202.0						230.0	17.55			
45	20,400	207.0	17.28	10,040	211.0	17.37	909			754	187.0			181.0	17.75
56				15,200	151.0	15.88					183.0	16.64	660	163.0	15.29
57				10,680	159.0	12.92							581	171.0	16.02
58				12,300	194.0	16.11							577	146.0	14.64
59		153.0	13.56					175.5	14.32						
65	8,380	78.0	6.83				1,182	180.0	14.11						
68	10,310	93.0	7.18												
		94.0	7.28												
120													1,065		17.76

Postmortem findings

Gross findings were not important.

Microscopic examination—

Heart. The moycardium of both dogs is normal. In dog 4 there is marked dilatation of the lymphatics of the epicardium. Some of them contain red blood corpuscles. One small artery in the myocardium shows a lesion of the media. Many smooth muscle fibers are densely stained, with pyknotic nuclei. Some of the smooth muscle cells are hyalin and acidophilic. Others stain densely with basic stain and are fragmented. In the adventitia there are many polymorphonuclear leukocytes.

Lungs. There are no lesions in the lungs of dogs 2 and 4.

Spleen. Dog 2. The follicles are small and inactive. The reticular cords contain very few lymphoid cells. In the sinuses are occasional clumps of densely stained fused red blood corpuscles. There is no erythropoiesis; no megakaryocytes. There are numerous phagocytes containing light yellow globules. The spleen of dog 4 shows some erythropoiesis and numerous megakaryocytes. The sinuses contain clumps of fused red blood corpuscles, as in dog 2. The secondary follicles show moderate activity.

Liver. In dog 2 there are many small to very large necroses. The smallest necroses appear in the mid-zonal regions. The larger necroses involve several lobules. These necroses contain very few leukocytes and are therefore regarded as of recent origin. There is fairly marked central fat vacuolization in the lobules throughout the liver. The cells lining the sinusoids are swollen and finely vacuolated. The liver of dog 4 shows no necrosis, no central fat vacuolization. The cells lining the sinusoids are swollen and vacuolated, as in dog 2. The liver cells as a whole contain more basic staining granular material than those of dog 2. In the sinusoids are occasional clumps of fused red blood corpuscles.

Gall bladder. The gall bladder from dog 2 is not preserved. That of dog 4 shows lesions in many arteries throughout the thickness of the gall bladder wall of the same nature as the lesions in the artery of the myocardium. These vascular lesions will be described more fully below.

Pancreas. The pancreas from each dog is negative. In dog 4 the larger veins contain clumps of fused red blood corpuscles.

Gastrointestinal tract. Dog 2. Esophagus, stomach and small intestine, normal. In dog 4 many blood vessels of the stomach

contain clumps of fused red blood corpuscles. In the muscularis there is one medium-sized vein with completely degenerated smooth muscle cells in the media, similar to the lesions of vessels of heart and gall bladder. Otherwise the stomach is normal. Small and large intestine normal.

Kidneys. The kidneys from both dogs are essentially normal except for atrophy of the epithelium of many of the distal convoluted tubules, more marked in dog 4. In dog 4 an occasional tubule shows the epithelium to be represented by a very narrow fringe of cytoplasm surrounding nuclei.

Adrenals. The adrenals from dog 2 are normal in appearance. Those from dog 4 show slightly less lipid vacuolization of the fascicular cells of the cortex.

Bladder. The bladder of dog 2 is normal except for a completely organized thrombosed vein on the external surface. The bladder of dog 4 is normal except for many acute vesicular lesions to be described below.

Genitalia. Testis—Dog 2. Immature, normal. *Uterus*—Dog 4. The mucosa is immature but otherwise negative. *Ovary*—Immature but otherwise negative.

Lymph nodes. The lymph nodes taken from many sources in both dogs show sinuses containing many red blood corpuscles. All of the lymph sinuses are dilated. There is only a moderate amount of phagocytosis of the red blood corpuscles. The lymphoid follicles are normal. Lymph sinuses and veins show clumps of fused red blood corpuscles. In one lymph node, from dog 4, there is an artery showing the degenerative lesion of the media noted in other arteries.

Thyroid gland. From both dogs, normal.

Skeletal muscle. That from dog 2 shows no lesion. That from dog 4 shows one vein with degeneration of the smooth muscle cells of the media.

Aorta. That from dog 4 is normal.

Skin. That from dog 2 is normal.

Vascular lesions in dog 4.[2] Degenerative lesions of the media are found in arteries and veins of the myocardium, gall bladder, urinary bladder, and one lymph node. These lesions are characterized by degeneration of smooth muscle cells and various stages in their development are present. The earliest change ap-

[2] The occurrence of clumps of fused red blood corpuscles in the sinusoids of the liver and sinuses of the spleen as an almost constant feature of hypervitaminosis A in rats and guinea pigs should be mentioned here as an early unpublished observation of one of the present authors (S. B. W.).

pears as an homogeneous dense acidophilic staining of the smooth muscle cells. More advanced lesions show infiltration of the media with strands of fibrin, proliferation of the intimal endothelium, and frequently a collection of red blood corpuscles beneath the endothelium. Mitotic figures are present in the adventitia and also in the endothelium. Some small veins show marked thickening as the result of intimal and adventitial proliferation. In such veins, while the lumen remains open, it is narrowed as a result of endothelial proliferation.

Another finding is that of mural thrombi of fused red blood corpuscles. The appearance suggests that the proliferation of the endothelium is the result of these thrombi, inasmuch as many of the thrombi are covered with one or more layers of endothelial cells. Occasionally red blood corpuscles are found intermingled with the proliferated (fibroblastic) cells of the adventitia. In some sections of the wall of the bladder practically every blood vessel shows these lesions. The lesions of the blood vessels of the gall bladder are identical and, also, in the mucosa there are many thrombosed small vessels which have resulted in small infarctions. In addition, many of the involved blood vessels are surrounded by small zones of hemorrhage.

While it seems unwarranted at present to regard these blood vessel lesions as a direct result of excessive vitamin A administration, we are at a loss to account for them, as they are quite different from any infectious lesion of the blood vessels that we know of and also quite different from the blood vessel lesions associated with the allergic reaction.

Bones. Based on knowledge of the skeletal changes resulting from vitamin A administration and gained from the study of small animals (Wolbach, '47), we selected for study in longitudinal and cross sections the epiphyseal ends of the tibia, fibula, femur, radius and ulna of dog 2. It should be kept in mind that the results on bone of excessive vitamin A administration are exhibited only in the growing animal and only in regions undergoing growth. A corollary from older studies is that the greater the rate of growth in the region the more marked are the consequences, which, in brief, consist of acceleration of the normal sequences of endochondral bone formation and the remodelling processes.

The effects on the growth sequences, including epiphyseal cartilage sequences and remodelling processes, are of the

same nature as those described for the rat and guinea pig (Wolbach, '47), though of lesser degree, which can be accounted for by the facts that the dogs were two months old when the experiment began and that the dosage of vitamin A was 300 I.U. per gram as compared to the 1,000 to 1,250 I.U. per gram used for rats and guinea pigs. The duration of the experiment on dog 2 was 68 days. With rats, the dosages used resulted, in 6 to 12 days, in the severe lesions including fractures already described (Wolbach, '47).

The epiphyseal cartilages in dog 2 of the regions chosen for study all exhibit complete consumption of fully grown cells, which has resulted in marked narrowing of the cartilage as a whole. Some of the appearances of the cartilage indicate that inanition effects have retarded the growth of the flattened cells in the cartilage columns, but here and there one sees deep penetration of the cartilage and blood vessels (fig. 1). Another indication of rapidity of growth sequences is the persistence of the bony trabeculae of the primary spongiosa.

In the shafts of the bones where remodelling normally takes place there are evidences of great increase in subperiosteal resorption of bone, just as has been described for smaller animals (figs. 2 and 3). There is, on the whole, much less evidence of new bone deposition, although newly deposited bone is present in regions of remodelling in conformity with the normal growth pattern and is much greater than deposition of bone in regions not being remodelled. The lesser amount of new bone formation as compared to rats and guinea pigs is probably the result of two factors—the slower tempo of the experiment and terminal inanition. In all regions of accelerated resorption of bone there is marked proliferation of periosteal cells and considerable hemorrhage, so that the appearances in such locations recall the hemorrhages of scurvy and a deficiency in intracellular matrix formation is to be considered as a possible factor, although it is not reconcilable with the appearances at the epiphyses and the deposit of osteoid wherever required by the normal growth pattern. Accelerated resorption of bone resulting in dimin-

ishing strength of periosteal attachments is a reasonable explanation of hemorrhages, although one we are not yet willing to commit ourselves to as a complete explanation because excessive vitamin A administration results in a depression of prothrombin levels (Light, Alscher and Frey, '44; Walker, Eylenburg and Moore, '47; Maddock, Wolbach and Jensen, '48).

Cross sections of femur, tibia and fibula reveal accelerated remodelling changes of cortical bone as required by the normal growth pattern. Cross sections of the femur show exaggeration of the flattening of the posterior surface with a great increase in the number of osteoclasts, periosteal proliferation and hemorrhage. The interior of the shaft in this region shows newly deposited osteoid and formation of compact bone in progress. The cortex, anteriorly, of the shaft shows a much less degree of the same process, indicating that at the level chosen the shaft is undergoing diminution in thickness.

The cross section of the tibia likewise shows remodelling sequences greatly in excess of the normal, most marked on the posterior surface and internal and anterior borders.

The most striking evidence of the accelerated remodelling is shown in the cross section of the fibula, of which only a small amount of compact bone remains. On one side, resorption is active. On the opposite side there is a thick layer of newly formed bone and osteoid, features required by the shift in relationships attending growth with increasing separation of tibia and fibula (figs. 4 and 5).

DISCUSSION

That characteristic skeletal changes and death as a result of the administration of excessive doses of vitamin A are phenomena present in the dog as well as in other mammals studied was doubted by some of the German workers in this field (Wendt, '35). The present study indicates that with sufficient dosage given over a sufficiently long time, the dog will respond as do the smaller laboratory animals. There is

some evidence to indicate that the dog, because of a possible lessened susceptibility to the effects of high doses of vitamin A, does not possess as delicately balanced a mechanism for the adjustment of vitamin A levels as does the rat or the human (Josephs, '42). The level of 2,420 I.U. per 100 ml of serum encountered during the course of this experiment in an adult dog on a regular diet is in excess of the maximum value of 950 I.U. per 100 ml of serum cited by Josephs in his human case of hypervitaminosis A (Josephs, '44), of the 1,200 reported by Toomey and Morissette ('47) and of the 2,081 reported by Rothman and Leon ('48) in other human cases. The dog is also unusual in his failure to respond to vitamin A administration by heightened cholesterol and phospho-lipid values, a finding noted by Wendt in 1935. Serum lipid rises after vitamin A feeding, either in large or toxic doses, have been noted in the human (Takahashi et al., '25; Harris and Moore, '28; Collazo, Rubino and Varela, '29); the rabbit (Harris and Moore, '28; Bomskov and Seemann, '33); the guinea pig (Collazo and Rodriguez, '33) and the rat (Drigalski, '33). The opposite effect, a fall in cholesterol (Drigalski, '33) and total lipids (Moll et al., '33) in the rat on vitamin A deficient diets, makes the implication that vitamin A is a factor in lipid metabolism a possible hypothesis.

The present study also emphasizes the fact that there are no detectable changes from the standpoint of the blood picture in carbohydrate, nitrogen and mineral metabolism. Except for a slightly heightened calcium level in one dog, the calcium, phosphorus and phosphatase values were essentially normal. Similar results have been noted in the rat (Davies and Moore, '34; Strauss, '34–'35; Cornil, Chevallier and Paillas, '39). Josephs' ('44) case of hypervitaminosis in a child showed normal calcium and phosphorus values but heightened phosphatase values, persisting as long as 6 months after the vitamin had been withheld.

In view of the great skeletal changes resulting from excess vitamin A administration, the absence of changes in calcium, phosphorus and phosphatase values may seem perplexing.

It must be kept in mind that the effect upon the skeleton is one of increased tempo of growth processes in complete conformity with normal growth patterns, in which removal of bone is balanced by new formations of bone and, *a priori*, the rate of metabolic activities resulting therefrom need not be reflected quantitatively in the blood.

It may be suggested that the accelerated rate of consumption of epiphyseal cartilage—essentially an acceleration of skeletal ageing— is accompanied by quantitative changes in the blood of an unknown nature.

SUMMARY AND CONCLUSIONS

1. The dog responds, as do other laboratory animals, to excessive doses of vitamin A.

2. This response is marked by loss of appetite, marked loss in weight, characteristic skeletal changes, hyperesthesia and exophthalmos.

3. Laboratory findings, with the exception of marked increases in the vitamin A content of the blood and a late drop in blood lipids (lipid-P and cholesterol), show no significant alterations.

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LITERATURE CITED

BLOOR, W. R., K. F. PELKAN AND D. M. ALLEN 1922 Determination of fatty acids (and cholesterol) in small amounts of blood plasma. J. Biol. Chem., *52:* 191.

BODANSKY, A. 1932 Determination of inorganic phosphate. Ibid., *99:* 197.

BOMSKOV, C., AND G. SEEMANN 1933 Über eine Wirkung des Vitamin A auf den Mineralhaushalt. Zschr. ges. exp. Med., *89:* 771.

BUTLER, A. M., AND E. TUTHILL 1931 An application of the uranyl zinc acetate method for the determination of sodium in biological material. J. Biol. Chem., *93:* 171.

CLAUSEN, S. W., W. S. BAUM, A. B. MCCOORD, J. O. RYDEEN AND B. B. BREESE 1940 Mobilization of vitamin A from its stores in tissues by ethyl alcohol. Science, *91:* 318.

COLLAZO, J., AND J. S. RODRIGUEZ 1933 Hypervitaminose A. Klin. Wschr., *12:* 1732.

COLLAZO, J. A., P. RUBINO AND B. VARELA 1929 Hypervitaminose D. Knochenbildung u. Wachstumsstörungen bei Ratten nach grossen Dosen bestrahlten Ergosterins. Deut. med. Wschr., *55:* 1794.

CORNIL, L., A. CHEVALLIER AND J. E. PAILLAS 1939 Etude histologique des lesions experimentales de la survitaminose A chez le cobaye. Ann. anat. path., *16:* 74.

DAVIES, A. W., AND T. MOORE 1934 Vitamin A and carotene. The distribution of vitamin A in the organs of the normal and hypervitaminotic rat. Biochem. J., *28* (1): 288.

DRIGALSKI, W. v. 1933 Über Schädigung durch Vitamin A. Klin. Wschr., *12:* 308.

FISKE, C. H., AND E. T. ADAMS 1931 The determination of calcium by alkalimetric titration. J. Am. Chem. Soc., *53:* 2498.

FISKE, C. H., AND Y. SUBBAROW Lipid phosphorus determinations. (Unpublished observations.)

FOLIN, O. 1934 Laboratory Manual of Biological Chemistry. D. Appleton-Century Co., Inc., New York, N. Y., 5th ed.

FOLIN, O., AND H. MALMROS 1929 An improved form of Folin's micro-method for blood sugar determinations. J. Biol. Chem., *83:* 115.

HARRIS, L. J., AND T. MOORE 1928 "Hypervitaminosis" and "vitamin balance." Biochem. J., *22* (2): 1461.

JOSEPHS, H. W. 1942 Factors influencing level of vitamin A in blood of rats. Bull. Johns Hopkins Hosp., *71:* 253.

———— 1944 Hypervitaminosis and carotenemia. Am. J. Dis. Child., *67:* 33.

LIGHT, R. F., R. P. ALSCHER AND C. N. FREY 1944 Vitamin A toxicity and hypoprothrombinemia. Science, *100:* 225.

LOWRY, O. H., AND O. A. BESSEY 1945 Microchemical methods for nutritional studies. Fed. Proc., *4:* 268.

MADDOCK, C. L. 1942 (Unpublished observations.)

MADDOCK, C. L., S. B. WOLBACH AND D. JENSEN 1948 Hypoprothrombinemia as a cause of death in hypervitaminosis A. Fed. Proc., *7:* 275.

MAY, C. D., K. D. BLACKFAN, J. F. MCCREARY AND F. H. ALLEN, JR. 1940 Clinical studies of vitamin A in infants and in children. Am. J. Dis. Child., *59:* 1167.

MIRSKY, I. A., AND M. BRUGER 1932 The effect of temperature on cholesterol determinations. J. Lab. Clin. Med., *18:* 304.

MOLL, T., O. DALMER, P. DOBENECK, G. DOMAGK AND F. LAQUER 1933 Über das Vitamin A-Konzentrat "Vogan." Arch exp. Path., *170:* 176.

MOORE, T., AND Y. L. WANG 1945 Hypervitaminosis A. Biochem. J., *39:* 222.

NOETZEL, H. 1939 Morphologische Untersuchungen bei Vitamin A Überdosierung. Zschr. ges. exp. Med., *105:* 83.

RODAHL K., AND T. MOORE 1943 Vitamin A content and toxicity of bear and seal liver. Biochem. J., *37:* 166.

ROTHMAN, P. E., AND E. E. LEON 1948 Hypervitaminosis A. A report of two cases in infancy. Radiology, *51:* 368.

STRAUSS, K. 1934–35 Beobachtungen bei Hypervitaminose A. Beitr. path. Anat., *F. 94:* 345.

TAKAHASHI, NAKAMIYA, KAWAKAMI AND KITASATO 1925 Scientific papers, Inst. Phys. Chem. Res. (Tokyo), *3:* 81.

TOOMEY, J. A., AND R. A. MORISSETTE 1947 Hypervitaminosis A. Am. J. Dis. Child., *73:* 473.

WAKERLIN, G. E., AND W. G. MOSS 1943 Treatment of experimental renal hypertension with vitamin A concentrate. Proc. Soc. Exp. Biol. Med., *53:* 149.

WALKER, S. E., E. EYLENBURG AND T. MOORE 1947 The action of vitamin K in hypervitaminosis A. Biochem. J., *41:* 575.

WENDT, H. 1935 Beiträge zur Kenntnis des carotin und vitamin A Stoffwechsels. Klin. Wschr., *14:* 9.

WESLAW, W., B. WRONSKI, A. WROBLEWSKI AND B. WROBLEWSKI 1938 Symptomalogie u. Verlauf der A-Hypervitaminose bei Ratten infolge enteraler, subcutaner u. percutaner Darreichung von Vitamin A Konzentraten. Ibid., *17:* (1) 777, 879.

WHITEHORN, J. C. 1920–21 Simplified method for the determination of chlorides in blood or plasma. J. Biol. Chem., *45:* 449.

WOLBACH, S. B. 1947 Vitamin A deficiency and excess in relation to skeletal growth. J. Bone Surg., *29:* 171.

————(See also: 1946 Vitamin A deficiency and excess in relation to skeletal growth. Ludvig Hektoen Lecture. Proc. Institute of Medicine of Chicago, *16:* No. 4, April 15, 1946.)

WOLBACH, S. B. AND O. A. BESSEY 1942 Tissue changes in vitamin deficiencies. Physiol. Rev., *22:* 233.

PLATE 1

EXPLANATION OF FIGURES

1 Dog 2. Photomicrograph. Epiphyseal cartilage, proximal end of tibia. The evidences of accelerated growth sequences are illustrated by deep penetration of the cartilage by blood vessels, the character of the primary spongiosa, and the penetration of the zone of residual cells by blood vessels from the epiphyseal side.

2 Dog 2. Photomicrograph. Proximal ends of the fibula showing complete consumption of active zones of epiphyseal cartilage, resorption of the periosteal bone, and subperiosteal hemorrhage.

1

2

PLATE 2

EXPLANATION OF FIGURES

3 Dog 2. Photomicrograph. Distal end of the radius. High power detail of accelerated resorption of bone beneath the periosteum with regions of concurrent bone deposition internally.

4 Dog 2. Photomicrograph. Low power. Cross section of fibula. This shows only a remnant of old bone. The upper right hand border is undergoing resorption and in the lower left there is new bone deposition.

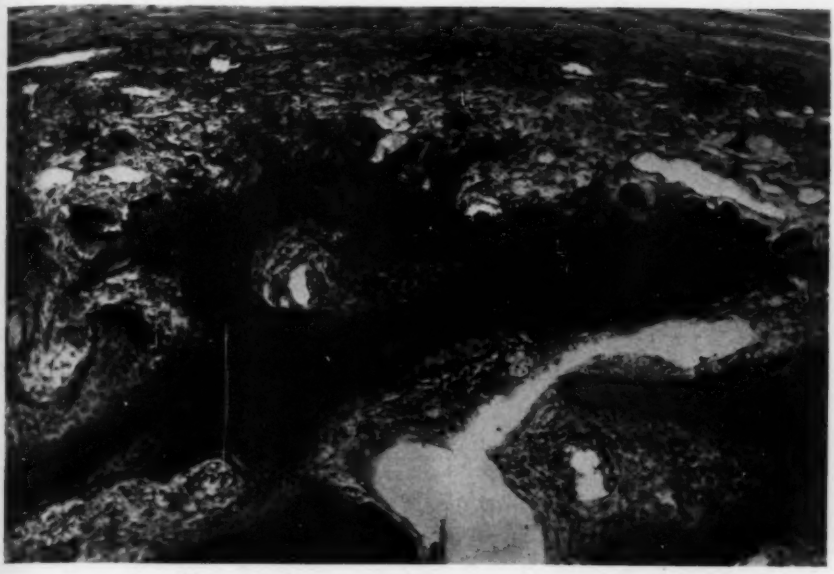

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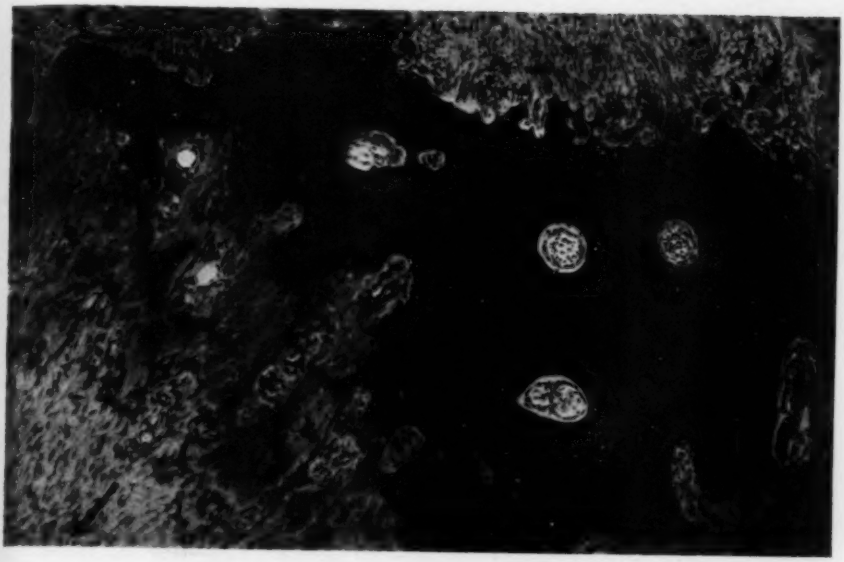
PLATE 3

EXPLANATION OF FIGURES

5 Dog 2. High power detail of figure 4, illustrating the accelerated compact bone formation in the fragment of old bone. Numerous osteoclasts on the site of resorption and accelerated appositional bone formation on the opposite side. In both figures 4 and 5 the arrows indicate the direction of movement of the fibula away from the tibia in conformity with the growth pattern.

6 Normal control dog. To illustrate the remodelling sequences in a normal dog. The arrow shows the direction of movement.



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